CHARACTERIZATION OF NUCLEAR MATRIX ALTERATIONS INVOLVED IN BLADDER CANCER PROGRESSION

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Bladder cancer, one of the most frequently diagnosed cancers, is a significant source of morbidity and mortality throughout the world. According to the American Cancer Society (2005), approximately 63,210 new cases will be diagnosed in the United States and bladder cancer will account for nearly 13,180 deaths. The current standard for detection of bladder cancer relies on cystoscopy, an invasive procedure, and cytology. Cytology has a high specificity, but lacks sensitivity in detection of low-grade tumors, as well as requires a trained pathologist for review. Because current diagnostic tools are less than optimal and because bladder cancer has a high rate of recurrence and long term monitoring is a necessity, a better diagnostic tool is needed. There is now a great interest in researching urine markers for bladder cancer.

Our lab previously identified six nuclear structural proteins (BLCA 1-6) that are specifically expressed in bladder cancer tissue. The nuclear matrix is the support scaffold of the cell nucleus. This structure has a variety of functions, many of which have implications in cancer progression.

The purpose of this dissertation is to examine changes in nuclear structural proteins. The hypothesis we propose is that changes in structural elements of the nucleus are involved in the

progression of bladder cancer and can be developed into markers of this disease. Specifically this study had three goals. 1) to determine if BLCA-1 could be developed into a biomarker of bladder cancer, 2) to clone the gene encoding BLCA-1, and 3) to examine functional aspects of BLCA-4.

A urine-based immunoassay was developed that can detect BLCA-1 in patients with bladder cancer with a specificity of 87% and sensitivity of 80%. Furthermore, this protein can be detected in serum of individuals with bladder cancer and may associate with the stage of disease. We also demonstrated that BLCA-4 can confer a growth advantage to cells over-expressing this protein. Over-expression of BLCA-4 led to many gene expression changes. BLCA-4 may play a role in bladder cancer pathobiology by altering genes that enhance proliferation and invasion, maintain blood flow for tumor cell survival, or enhance angiogenesis. Finally, we have been successful in cloning part of the cDNA that encodes for BLCA-1 and it appears to have a close homology to a novel metastasis related gene.

In summary, this project has demonstrated that bladder cancer specific nuclear matrix proteins can be developed into markers of the disease and may play a functional role in bladder cancer pathobiology.

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PREFACE

I would like to begin by first thanking my thesis advisor, Robert Getzenberg. He has been a wonderfully supportive and encouraging mentor, whom I have learned so much from and have a great respect for. I can only hope that I have made as lasting an impression on him as he has on me. We've had some good laughs, haven't we? By the way Robert, I want to remind you that this is a thesis written by a York County native and York College graduate! I can't imagine that I would have enjoyed my time in the lab any more than I have in the Getzenberg lab for the past five years. I have learned so much, had such fun, and made wonderful friends during my time there. I would like to thank everyone that was a part of the lab while I was there for all of their help and friendship, especially the "ladies of the lab". We had many people come and go during my time there, but they have all influenced me in some way. I can't forget other members of the department either. The Urology department is a special place with special people who all have come to mean a great deal to me.

I would also like to thank the members of my committee, Dr. DeFranco, Dr. Pflug, Dr. Birder, Dr. Godfrey, and Dr. Yoshimura for their participation. I would like to mention a special thanks to Dr. Pflug who took me under her wing and guided me after Robert left the university.

I can't thank my family and friends enough for their support through this process. Some of those friends I have made as a result of this program and who have become extremely special to me, while others are old friends that have stuck by me for years. I especially need to thank my

parents without whom I would not be here. I could not ask for any more support from them than they have given me over the years. Constantly encouraging, helpful, guiding, and loving, I would not have made it without them. I've come a long way, haven't I mom and dad? Also, thanks to my sisters who have always been there for me and can always cheer me up. Finally, I have to thank my husband Patrick, who has been with me throughout the entire process. He sticks by me through ups and downs, tears and smiles. I could not ask for a more supportive, loving or sweet person to spend my life with.

The years of graduate study have certainly changed me. I have become a more independent person and more independent thinker, and I am taking away a lifetime of life lessons and memories from this process.

1. INTRODUCTION

1.1. Cancer

Cancer is the second leading cause of death in the United States. It is estimated that over 570,000 individuals will die of these diseases this year (ACS 2005). Because of its huge impact on society, there are enormous research efforts to better diagnose, treat, and understand these diseases. Cancer is a term for a large number of diseases that are characterized by changes in a cell that leads to uncontrolled cell growth. Normal and cancer cells undergo the same differentiation, division, and apoptotic mechanisms; however, the regulation of these functions differs between these cells.

Cancer can be triggered by either exposure to environmental factors (tobacco, chemicals, radiation), genetic predisposition, or a combination of the two (ACS 2005). It is estimated that approximately 75% of all cancers are caused by environmental factors. Smoking is linked to at least 10 different cancers and accounts for 30% of cancer deaths. Environmental exposure to certain chemicals such as benzene, asbestos, and lead cause a variety of tumors. Radiation, or the emission of energy from any source, can cause mutations in DNA which can lead to cell death or cancer. Ionization is one of the few scientifically proven carcinogens in humans (ACS 2005). While individuals can try to minimize their cancer risk by altering their lifestyle, cancer is not always preventable. Therefore, screening for and early detection of cancer are extremely important.

1.2. Bladder Cancer Statistics

Bladder cancer is a significant source of morbidity and mortality in the United States. According to the 2005 American Cancer Society statistics, 63,210 total cases of bladder cancer will be diagnosed this year, and 13,180 total deaths will occur because of this disease. It is the fourth most common cancer in males and the 10th in women, accounting for 6% and 3% of total cancer cases respectively. Bladder cancer is a predominately male disease, with males being diagnosed approximately four times more often than females, and as many as 1 in 29 males will be diagnosed with bladder cancer in their lifetime (ACS 2005). Bladder cancer is typically a disease of the elderly, and the frequency of this disease increases with age (Campbell, et. al 2002).

1.3. Risk Factors for Bladder Cancer

It is still unknown exactly what causes bladder cancer. However, researchers have found some risk factors for developing the disease and are exploring how these factors cause cells to become cancerous. Some of the most common risk factors for bladder cancer include smoking, exposure to chemicals such as aniline dyes and aromatic amines (Campbell, et al. 2002), and chronic inflammation of the bladder (Kantor, et. al 1984). Smoking and exposure to arylamines alone are thought to contribute to over 50% of all bladder cancer cases (Moyad 2003).

1.4. Symptoms of Bladder Cancer

There are a few symptoms of bladder cancer, although they are not effective for diagnosis of the disease. The most common symptom seen in up to 85% of patients is hematuria, or the presence of blood in the urine. However, only 2-4% of patients that have microhematuria actually have bladder cancer, and only approximately 25% of people that present with macrohematuria have bladder cancer (Campbell, et al. 2002). Therefore, over 90% of patients that exhibit hematuria do not have bladder cancer. Other symptoms of bladder cancer include painful or increased frequency of urination or abdominal pain, but these symptoms are also indicative of diseases other than bladder cancer (Van Le, et. al 2004).

1.5. Types of Bladder Cancer

In the United States, more than 90% of bladder tumors are transitional cell carcinomas (Al Sukhun, et. al 2003), or cancers of the urothelial cells that line the bladder. Characteristics of these tumors include an increased number of epithelial cell layers, loss of cell polarity, increased nuclear-cytoplasmic ratio, prominent nucleoli, clumping of chromatin, and increased number of mitoses. This type of tumor has great potential for metastasis (Campbell, et al. 2002).

Squamous cell carcinomas of the bladder are seen in about 5-6% of cases, and adenocarcinomas represent 1% of bladder tumors (Vogelzang, et. al 1996). Squamous cell carcinomas are often seen in Egypt, where there are common infections with *S. haematobium*. Squamous cell cancers also arise from chronic infections and indwelling catheters, and these tumors are more common in circumstances where the bladder is chronically inflamed. In fact, there have been numerous reports examining the association of bladder cancer and people with

long-term catheterization due to spinal cord injuries. Possible suggested causes of this increased development of bladder cancer in patients with spinal cord injuries are chronic irritation by a catheter, the presence of bladder stones, and recurrent urinary tract infections (Subramonian, et. al 2004). Some reports have suggested that patients with spinal cord injuries have up to a 460 times higher incidence of bladder cancer compared to the general population (Konety, et. al 2000). Therefore, this population is a high-risk population and for that reason a unique cohort in which to study this disease.

1.6. Stages and grades of Bladder Cancer

Bladder tumors are commonly described with a grade and stage. Tumor grading is a pathological designation while staging is a clinical designation. Grade one pathological tumors are well-differentiated tumors and are described to have more than the normal seven urothelial cell layers and only have slight anaplasia and pleomorphism. Often, this grade of tumor is found in bladders that also have higher-grade tumors. Grade two tumors are moderately differentiated and have loss of cell polarity, a high nuclear-cytoplasmic ratio, more pleomorphism, and prominent nucleoli. Grade three tumors are poorly differentiated with cells that either did not differentiate or have de-differentiated, a high nuclear-cytoplasmic ratio, and marked nuclear pleomorphism (Campbell, et al. 2002). In 1998 the World Health Organization and the International Society of Urological Pathology proposed a new classification for tumor grading. Grade 1 tumors are referred to as papillary urothelial neoplasms of low malignant potential, grade 2 tumors are referred to as low grade urothelial carcinomas, and grade 3 tumors are named high-grade urothelial carcinomas (Lopez-Beltran, et. al 2004).

Among high-grade tumors is a type of tumor referred to as carcinoma in situ. This is a poorly differentiated transitional cell carcinoma that is confined to the urothelium and is pathologically a very flat lesion. This type of cancer appears in anywhere from 20-75% of high-grade muscle invasive tumors, and carcinoma in situ correlates with muscle invasive disease. It is therefore thought that it is a precursor to invasive bladder cancer (Campbell, et al. 2002).

Another tool used when describing a bladder tumor is tumor staging. Tumor staging describes the spread of the cancer and is most often described using the TNM (tumor-node-metastasis) system. This system relies on the pathologist to diagnose the depth and extent of invasion. Figure 1 and table 1 provide visual and text descriptions of the tumor staging (Lopez-Beltran, et al. 2004).

Table 1: TNM staging of the bladder (Lopez-Beltran, et al. 2004)

Primary Bladder Tumor Stages (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Papillary no-invasive carcinoma
Tis	Carcinoma in situ
T1	Tumor invades sub-epithelial connective tissue
T2	Tumor invades muscle
T2a	Tumor invades superficial muscle
T2b	Tumor invades deep muscle
T3	Tumor invades perivesical tissue
T3a	Microscopic invasion
T3b	Macroscopic invasion
T4	Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, or abdominal wall
T4a	Tumor invades prostate, uterus, or vagina
T4b	Tumor invades pelvic or abdominal wall

Lymph Nodes (N)

NX	Regional Lymph nodes cannot be assesed
N0	No regional lymph node metastasis
N1	Metastasis in single lymph node, 2 cm or less in dimension
N2	Single lymph node metastasis more than 2 cm but less than 5 cm, or multiple lymph nodes, none greater than 5 cm
N3	Metastasis in lymph node greater than 5 cm

Distant Metastasis (M)

MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

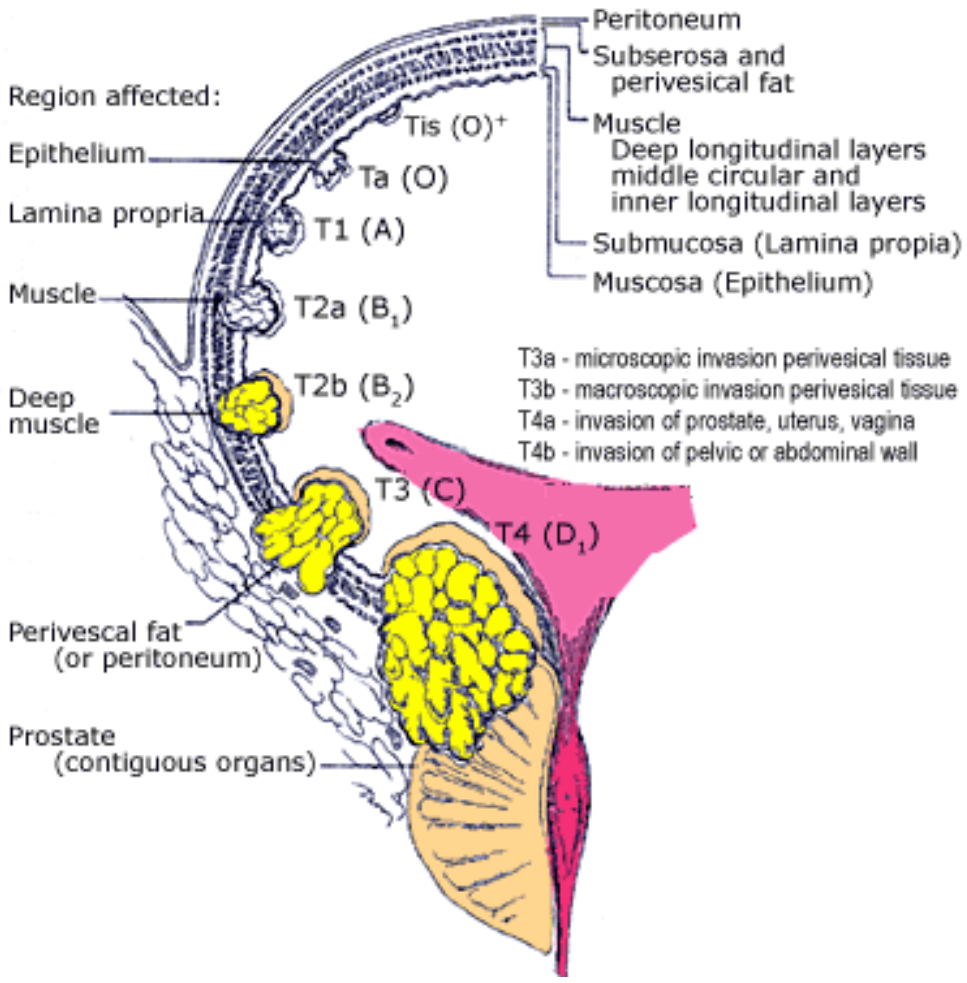

Figure 1: Illustration of tumor stages

(http://www.biologictherapy.org/bladdercancer/bladder_staging_tnm.html)

1.7. Molecular Genetics of Bladder Cancer

There are a number of functions that can be dysregulated in a cancer cell. One such altered mechanism is a change in chromosome and genetic organization, which can lead to aberrant expression of various genes. The introduction of microarray analysis has opened up many new doors to explore genetic changes occurring in cancer cells. Genetic analysis shows that multitudes of genes are either up or down regulated in human cancers. Some common genes that undergo expression changes are p53, Rb, and cyclin D1 (Bast, et. al 2000). The tumor suppressor gene (a gene that reduces the probability that a cell will turn into a tumor cell), p53, is a major player in cell cycle control and has been found to be mutated in most tumor types. The normal role of the p53 pathway is to arrest cells in G1 allowing cells to repair DNA damage before it is replicated in S phase (Dean, et. al). This defect in the cell cycle permits cells to continue past cell cycle checkpoints and allow cells to more readily accumulate mutations. Changes in gene expression often provide targets for therapy or cancer detection.

No specific genetic alteration has been identified that is responsible for the initiation of bladder cancer. Multiple genetic events may lead to the development and progression of bladder cancer, including loss of genetic material from tumor suppressors, over-expression of genes, translocations, and gene mutations. The majority of genetic events shown to occur in bladder cancer are associated primarily with loss of genetic material from tumor suppressors genes, leading to loss of heterozygosity of numerous genes (Sandberg 2002). Examination of these genetic events may provide insight into the cause of bladder cancer as well as tools for diagnosis, prognosis, and therapy for this disease. A number of the most common genetic abnormalities that have been identified in bladder cancer will be discussed below.

Multiple studies have examined chromosomal abnormalities in bladder cancer, and it appears that chromosome 9 plays a key, if not primary, role in bladder cancer pathogenesis (Sandberg 2002; Al Sukhun, et al. 2003). Loss of heterozygosity on chromosome 9 appears to lead predominantly to Ta-T2 tumors, while additional genetic events lead to progression of the disease to later stages. Three tumor suppressor genes, p14, p15 and p16 are located on 9p21. These genes normally regulate the cell cycle so their loss can lead to uninhibited proliferation and therefore may have a potential role in bladder cancer (Sandberg 2002). p15 and p16 proteins bind to cyclin-dependent kinases and inhibit phosphorylation of pRb, which in turn halts cells at the G1 phase. Loss of the p15 or p16 genes can therefore lead to uncontrolled progression through the cell cycle. It has been fairly well established that loss of chromosome 9 is the initial genetic event responsible for the onset of abnormal bladder cell proliferation, but the subsequent genetic events are not as well established.

Deletions of chromosome 11 have also been documented in bladder cancer. LOH of chromosome 11 has been implicated in approximately 30% of invasive bladder cancers but is rare in superficial tumors. It is therefore thought that deletions of this chromosome are a later genetic change during the progression of bladder cancer. p57 is a cell cycle inhibitor located on chromosome 11. Studies have found that p57 is often downregulated in TCC (Hoffmann, et. al 2005).

While loss of chromosome 9 appears to lead to low-grade superficial tumors, high-grade cancers are more commonly associated with loss of chromosome 17 (Sandberg 2002; Al Sukhun, et al. 2003). Deletions of chromosome 17 are not seen in superficial noninvasive papillary tumors, while 60% of invasive tumors have LOH of this chromosome, suggesting an involvement in progression of bladder cancer (Al Sukhun, et al. 2003). The p53 gene resides on

chromosome 17. As mentioned earlier, p53 functions to initiate apoptosis in cells with DNA damage and suppress cell proliferation and is the most frequently modified gene in human cancers. One of the roles of p53 is to bind to DNA, which then induces the production of p21 protein. p21 binds to cdk2, a protein that stimulates cell division, and this complex arrests cells in G1 allowing cells to repair DNA damage before it is replicated in S phase (Dean, et al.). Mutations in p53 allow cells to more readily accumulate mutations.

An additional function of p53 includes the induction of thrombospondin-1, which is an inhibitor of angiogenesis (Dean, et al.). Tumors require new blood vessels in order to grow and sustain themselves. Therefore, if p53 is mutated or missing an important angiogenesis inhibitor will not be induced and tumor vasculature can advance. Studies have shown that mutations in p53 are significantly associated with bladder cancer stage and grade (Erill, et. al 2004; Lorenzo Romero, et. al 2004). Deletion of the 17p region, the region where p53 is located, is also significantly associated with bladder tumor stages and grades (Erill, et al. 2004). p53 levels can be measured in the serum of patients using an ELISA. Serum p53 levels are significantly higher in bladder cancer patients than controls and levels are significantly higher in invasive tumors than in superficial tumors (Malviya, et. al 2004). While this may not be a diagnostic marker of bladder cancer since p53 is altered in so many cancer types, it may be a marker of bladder cancer invasiveness. Deletions or mutations of the p53 gene promote further genetic instability and selection for aggressive behavior of cells. While p53 mutations are common in bladder cancer, approximately 50% of metastasizing tumors show no alterations in p53 indicating that multiple pathways must be involved in tumor progression (Al Sukhun, et al. 2003).

Another gene commonly altered in a multitude of cancers, including bladder, is the Rb gene, located on chromosome 13. The Rb gene product functions as a brake in the cell cycle.

When the Rb protein is underphosphorylated, Rb binds to gene regulatory proteins and prevents them from acting in the nucleus to promote DNA replication. However, when Rb is hyperphosphorylated cell cycle progression can occur (Alberts 2002). Loss of Rb results in cells continuing through the G1 to S phase checkpoint, thus stimulating cell proliferation. Various groups have examined Rb expression in bladder cancer. It has been reported that both Rb mRNA and protein levels are altered in patients with bladder cancer. Furthermore, a decrease in Rb is related to tumor stage, tumor grade, and disease-specific survival (Shariat, et. al 2004). Another group has demonstrated that Rb can be inactivated by hypophosphorylation in bladder cancer (Chatterjee, et. al 2004). This hypophosphorylation can occur as a result of loss of p16 expression, which has been exhibited in bladder cancer, as discussed earlier. Alteration of chromosome 13 and Rb is observed more often in aggressive transitional cell carcinomas and is hypothesized to be another mechanism by which bladder cancer can progress (Sandberg 2002).

Hypermethylation of the promoter regions of genes can also occur frequently in various tumors. Hypermethylation can lead to transcriptional silencing and therefore down-regulate gene expression. It has been reported that some genes, including the tumor suppressors APC, RASSF1A, and p14, are hypermethylated in bladder cancer (Dulaimi, et. al 2004). Methylation of various genes correlates with high-stage, high-grade, muscle invasion, and shortened survival (Dean, et al.). Consequently, methylation of genes may be a prognostic indicator of bladder cancer.

The genetic alterations discussed here are just a few of the more common ones that occur in the progression of bladder cancer, but is by no means a comprehensive compilation of the genetic events that lead to this disease. While some possible genetic pathways to bladder cancer development have been proposed, there is still much to learn. With the development of DNA

microarrays, improved classification of bladder tumors and the genetic events involved in bladder cancer initiation and progression will be forthcoming.

1.8. Diagnostics

At the time of diagnosis with bladder cancer, about 75% of patients have superficial bladder cancer, with tumors confined to the mucosa or lamina propria. Superficial bladder cancer has a very high rate of recurrence with up to 70% of patients suffering from recurrence of this disease, and 10% to 30% will present with grade and stage progression. These tumors can recur even years after therapy, so long term surveillance of patients is necessary. When this cancer is detected at a localized stage the 5 year survival rate is 94% (ACS 2005). It is estimated that between 5-20% of patients with superficial tumors will develop invasive or metastatic disease. Approximately 30% of bladder cancer cases are muscle invasive or have metastasized at initial presentation. Unfortunately 50% of these patients will die within 2-3 years of diagnosis even after aggressive treatment (Kwak, et. al 2004). Patients that have high-grade tumors at the time of diagnosis are more likely to develop invasive and metastatic disease. The most common sites of metastatic spread are the pelvic lymph nodes, liver, lungs, bone, adrenal glands, and intestines and few patients will survive five years once their cancer has spread to distant sites (Campbell, et al. 2002).

Because of the high diagnosis rate and lack of symptoms for this disease, high rate of recurrence, and necessity of long term follow-up, a sensitive and specific detection tool that can be easily sampled is needed. Currently, the most commonly used diagnostic tools are cytology and cystoscopy. Cytology is the microscopic examination of cells that are shed into the urine. This method has been in use to detect bladder cancer for the last 60 years. Cytology has a very

high specificity, however, it lacks sensitivity in the detection of low-grade disease. While the specificity of this test is reported to have a mean of 95.7%, the mean sensitivity is only 49.1% (Konety, et. al 2004). It has even been reported that up to 20% of high-grade tumors can be falsely negative when using urine cytology (Campbell, et al. 2002). Again, a sensitive test that can detect these tumors at an early stage is critical for the survival of patients. Not only does this test have a low sensitivity, but also requires a trained pathologist for review.

Cystoscopy accompanied by a bladder biopsy is referred to as the gold standard for bladder cancer diagnosis. This is an invasive procedure that involves inserting a flexible scope into the urethra and visually looking for a tumor (Sengupta, et. al 2004). Because of the flat nature of carcinoma in situ, it is difficult to detect this tumor type using cystoscopy. This method can also be difficult to use when there is inflammation of the bladder or when an individual has an indwelling catheter. These are some shortcomings of the test, not to mention the discomfort and expense to perform it.

1.9. Bladder Cancer Treatment options

Most low-grade noninvasive tumors are treated with transurethral resection of bladder tumor (TURBT). This surgical procedure that is both diagnostic and therapeutic involves removal of the cancer. However, up to 70% of low grade bladder tumors will recur within a 5 year period after TURBT (Dinney, et. al 2004). Due to the high rate of recurrence, patients must be monitored regularly. After treatment, individuals must undergo a cystoscopic examination every three months for the first year, every six months for the second year, and then once a year annually after that (Sengupta, et al. 2004). Because of the high rate of occurrence, patients are often treated with intravesical chemotherapy with drugs such a mitomycin C and BCG. The

purpose of this treatment is three fold; to decrease recurrence, prevent progression, and eradicate residual disease after TUR. The most effective intravesical agent used today is Bacille Calmette-Guerin (BCG). It is an attenuated mycobacterium that has antitumor activity against multiple tumors. The exact mechanism of action is not known, but it appears to initiate a T-helper cell response. Mitomycin C is a cross-linking agent that inhibits DNA synthesis, but is less effective for treating bladder cancer (Campbell, et al. 2002).

Muscle invasive tumors (T2 and above) are usually treated by cystectomy, or surgical removal of the bladder and pelvic lymph nodes. Survival of these patients is dependent on disease stage (Sengupta, et al. 2004). This treatment is often curative for those patients with organ-confined, node-negative disease (Borden, et. al 2004). Therapy for localized disease could also include multimodality therapy. This is a combination of aggressive TUR, radiotherapy, and systemic chemotherapy. This allows patients to preserve the bladder, although many patients require subsequent cystectomy. This therapy does not allow the ability to provide pathologic staging, it requires strict patient compliance, and has the potential to be very costly. Compared to radical cystectomy, studies suggest comparable survival rates. Therefore, cystectomy remains the standard of care for patients with muscle invasive bladder cancer and bladder preservation should only be used in patients that are unable to undergo radical surgery (Borden, et al. 2004).

Approximately 50% of patients with muscle-invasive bladder cancer have recurrent distant disease after cystectomy (Borden, et al. 2004). Therefore, additional treatment may be necessary. Neoadjuvant chemotherapy (chemotherapy given before local treatment) using a combination of methotrexate, vinblastine, doxorubicin, and cisplatin (M-VAC) has been shown to be more effective than any single drug alone. Previously, the use of cisplatin-based therapy failed to demonstrate a therapeutic benefit for neoadjuvant chemotherapy (Campbell, et al.

2002). Recently however, an 11 year study examining survival after three cycles of M-VAC given prior to cystectomy found that the median survival of patients receiving chemotherapy was much higher than those treated with surgery alone. Additionally, more patients that received chemotherapy had no residual disease (Shah, et. al 2004). A separate study failed to show a clinically worthwhile benefit in the use of three cycles of cisplatin, methotrexate, and vinblastine before definitive treatment (Mead, et. al 2004). Therefore, the use of neoadjuvant chemotherapy is still questionable. A combination of gemcitabine and cisplatin (GC) has been compared to M-VAC therapy, and both therapies demonstrated similar overall survival. The GC therapy was better tolerated with less toxicity and is now considered to be an acceptable standard regimen for patients with metastatic or locally advanced bladder cancer (Borden, et al. 2004). The advantages of neoadjuvant therapy is that the response to chemotherapy may predict future clinical course, it allows for possible treatment of micrometastases without delay, and there is the potential of bladder preservation if the disease responds well to chemotherapy (Tsukamoto, et. al 2004).

The use of adjuvant therapy (chemotherapy given to patients who have received potentially curative treatment that are at high risk of recurrence or metastasis) is still under investigation. Adjuvant therapy allows for accurate stage diagnosis and there is no delay in performing cystectomy. However, there is no chance to preserve the bladder, there is no marker to assess the response to chemotherapy, and there is a delay for treatment of micrometastases (Tsukamoto, et al. 2004). The majority of studies examining the benefits of adjuvant therapy contained few patients and several published studies were criticized for early termination or use of inappropriate statistical analysis. There is a study currently underway to compare the use of

immediate postoperative chemotherapy versus chemotherapy that is deferred until relapse (Mead, et al. 2004). Table 2 reviews the current standard treatments for bladder cancer.

Table 2: Current standard treatments for bladder cancer (Schenk-Braat, et. al 2005)

Classification	Description	Treatment
Tis	Carcinoma in situ	Intravesical BCG, followed
	restricted to mucosa	by radical cystectomy if
		needed
Ta	Noninvasive papillary	TUR+adjuvant intravesical chemotherapy for
	carcinoma	low and intermediate risk tumors or adjuvant
		BCG for intermediate and high risk tumors
T1	Tumor invades subepithelial	TUR+adjuvant intravesical chemotherapy for
	connective tissue	low and intermediate risk tumors or adjuvant
		BCG for intermediate and high risk tumors
T2, T2a, T2b	Tumor invades muscle	Radical cystectomy
T3, T3a, T3b	Tumor invades perivesical tissue	Radical cystectomy
T4, T4a, T4b	Tumor invades neighboring organs	Radical cystectomy
N+ and	Metastases in regional lymph nodes,	Radical cystectomy + systemic chemotherapy
metastases	distant lymph nodes, and/or distant	
	organs	

1.10. Prognostic markers

Researchers have begun looking for molecular markers to assess prognosis of bladder cancers in individuals. In order for a marker to be of value, it must add predictive ability beyond the current clinical and pathologic parameters. Currently, the most prognostic factors are bladder cancer stage, grade, and presence of carcinoma in situ (Campbell, et al. 2002). As mentioned earlier, up to 70% of patients suffer recurrence of bladder tumors and as many at 30% will present with stage and grade progression (ACS 2005). It is difficult to predict exactly which tumors will recur and which will progress. Therefore, a reliable molecular marker that can predict disease prognosis and recurrence potential would have high clinical significance.

As discussed in the molecular genetics of bladder cancer section, the retinoblastoma tumor suppressor gene and p53 are altered in bladder cancer. A recent report used immunohistochemical analysis on patient samples, collected from patients who had previously undergone radical cystectomy for the treatment of bladder cancer, to examine the level of protein expression of p53 and Rb, along with p16 and p21. Over 80% of bladder cancer patients had alterations in at least one of these markers. The expression of p53 and pRB/p16 were associated with muscle invasive disease, but not the presence of carcinoma in situ, pathologic grade, lymph node metastases, or lymphovascular invasion. The altered expression of p53, p21, and pRB/p16 were each also significantly associated with an increased probability of tumor progression and decreased survival from bladder cancer. Because combining markers that work at least partly through independent pathways often provides better prognostic accuracy, they examined combinations of p53, p21, and pRB/p16. This research project found that the risk of experiencing bladder cancer progression as well as decrease in survival increased incrementally in proportion to the number of markers altered (Shariat, et al. 2004).

Likewise, in a similar study, p53, p21, and pRb expression was examined immunohistochemically on archival radical cystectomy samples from 164 patients with invasive or high-grade recurrent superficial transitional cell carcinoma. Each of these markers was an independent predictor of bladder cancer progression and survival. When these markers are used in combined analysis, alterations in two or three of the markers combined caused a significant decrease in survival and increase in recurrence. In fact, almost all patients that had alterations of all three markers suffered from recurrence and died within 5 years. Therefore, combined analysis of p53, Rb, and p21 provides strong prognostic abilities and may indicate the need for adjuvant therapeutic strategies (Chatterjee, et. al 2004).

While expression levels of p53 have been shown to be predictors of disease survival and recurrence, it is controversial whether expression correlates with response to BCG treatment. One report suggests that nuclear expression of p53 before BCG treatment was not able to predict how patients would respond to this therapy. However, p53 protein over-expression before BCG therapy did correlate with disease recurrence following this therapy. Additionally, p53 expression following BCG treatment is a prognostic marker of disease recurrence, although pre-therapy p53 levels are a stronger predictor (Lacombe, et. al 1996). Another study suggests that over-expression of p53, as determined immunohistochemically, has no predictive value for recurrence and progression in T1G3 bladder cancers treated with intravesical BCG (Peyromaure, et. al 2002). In yet another study, over-expression of p53 was inversely related to the response to BCG therapy (Lee, et. al 1997). Therefore, the use of p53 as a prognostic marker for response to therapy is still controversial and requires more research.

UroVysion has been approved by the FDA to aid in the diagnosis of bladder cancer and will be discussed further in the following gene based bladder cancer assays section. A study was performed to examine the utility of UroVysion (a FISH based assay) to predict patient response to BCG and other intravesical therapies. Fluorescence *in situ* hybridization (FISH) is used to detect chromosomal abnormalities in various cancers. The study demonstrated that patients with a positive post-therapy FISH result are significantly more likely to have tumor recurrence following therapy than patients with a negative post-therapy FISH result, and these recurrences occurred earlier. Results also showed that patients with a positive post-therapy FISH result were more likely to have muscle invasive cancer than patients with a negative post-therapy FISH result. Therefore, this may be a useful tool to aid in the determination of the response to intravesical therapy (Kipp, et. al 2005).

1.11. Gene Based Bladder Cancer Assays

As discussed earlier, changes in chromosomes often occurs in bladder cancer. FISH has been performed on urine samples from patients with bladder cancer to examine its utility as an alternative or in conjunction with the current tools. FISH is able to identify chromosome number abnormalities, particularly of chromosome 9 and 17, from urine samples. From this study, it appears that these abnormalities are not very prevalent in low grade and low stage tumors limiting their utility (Okamura, et. al 2004). Other reports suggest that FISH may have a higher sensitivity than cytology. In one study, 60% of patients with biopsy-proven TCC but negative cytology were successfully identified with FISH (Quek, et. al 2004). One study presented nine false-positive FISH results in patients with negative biopsies. Within 15 months, all nine patients had developed biopsy proven lesions (Skacel, et. al 2003), indicating that FISH may represent early genetic alterations that are predictive of later bladder cancer development.

The FDA has recently approved one gene-based test, UroVysion, to aid in the diagnosis of bladder cancer in patients presenting with hematuria. Previously, this test had FDA approval to be used along with cystoscopy to monitor for bladder cancer recurrence. UroVysion applies FISH techniques to urine samples. The UroVysion^{TM} Bladder Cancer Kit (UroVysion Kit) is designed to detect aneuploidy for chromosomes 3, 7, 17 and loss of the 9p21 locus. A large scale 23 site study of almost 500 patients revealed that this test has a sensitivity of 68.6% and a specificity of 77.7%. Urine cytology was also performed on these patients and in this study had a sensitivity of 39.2% and a specificity of 91.5% (Vysis 2005). While this test is the most sensitive in the detection of high grade and stage tumors, it is able to detect low grade and stage tumors with lower sensitivity (UroVysion 2005).

The National Cancer Institute is currently conducting a large-scale clinical trial to examine microsatellite analysis of urine sediment as a diagnostic tool for bladder cancer. Microsatellites are nucleotide repeats that are interspersed throughout the genome and the number of repeats varies between individuals. Microsatellite analysis to examine loss of heterozygosity of 15 markers is being performed (NCI 2005). A recent publication reports that microsatellite analysis of urine sediment (MAUS) using 16 markers has higher sensitivity than cytology for detection of low-grade tumors. MAUS had a sensitivity of 41% for stage Ta tumors while the reported sensitivity of cytology was only 23%. The sensitivity of both tests was 49% for stage T1-T4 tumors. This report also suggests that inflammation of the bladder does not result in microsatellite alteration indicating that results would not be affected by cystitis or other benign inflammatory responses (Fornari, et. al 2004). Another study compared normal DNA (extracted from peripheral blood mononuclear cells) with tumor DNA extracted from urine sediment of individuals with bladder cancer. They reported finding 91 microsatellite alterations in the DNA from urine, and using eight markers report a sensitivity of 87%. They also report a correlation between tumor grade and the frequency of microsatellite instabilities as well as a correlation between microsatellite instabilities and invasive tumors. This study did not report any alteration in the lowest grade tumors, indicating that this assay may have similar limitations as cytology (Berger, et. al 2002). While there are definite microsatellite instabilities that occur in bladder cancer, it appears that the selection of microsatellite markers to examine will play a large role in the success of this analysis.

Additionally, flow cytometry can be used to measure the DNA content of cells whose nuclei have been stained. Therefore, flow cytometry is able to quantitate the aneuploid cell populations and the proliferative activity of a tumor. Tumors that are DNA diploid tend to be of

low grade and stage and have a favorable prognosis. However, tumors with a triploid to tetraploid chromosome number have a poor prognosis. One drawback of this method is that interpretation can be difficult because inflammatory cells can form diploid or hyperdiploid cell fractions, leading to questionable prognosis. To date this assay has not been found to be more clinically valuable than cytology. Furthermore, low-grade tumors, which are often diploid, can produce false-negative results. However, high-grade tumors often display aneuploidy making flow cytometry very accurate under these conditions (Campbell, et al. 2002).

1.12. Biomarkers

A biomarker is a molecule that changes over time and indicates an alteration of the physiological condition of an individual in relation to health or disease state, drug treatment, or toxins (Zolg, et. al 2004). Biomarkers can be used as diagnostic tools or to measure the efficacy of drug treatments for disease. Validation of biomarkers requires extensive clinical trials in order to compare them with currently used and approved clinical tools. Disease states often have different protein expression than healthy states, and this differential expression can have high diagnostic utility. These specific proteins can be used as screening markers to differentiate between healthy and disease states, with the goal being early detection of the disease. Biomarkers can also be used as prognostic markers, meaning that they will predict the course of disease once it is established. These markers can be useful to influence the aggressiveness of therapy. Stratification markers can be used to predict the response of an individual to a specific drug treatment. Another type of biomarker is the efficacy marker, which is used to measure the efficacy of drug treatment (Zolg, et al. 2004). The field of proteomics includes the identification and study of proteins as biomarkers.

1.13. Proteomics

The field of proteomics, the study of the protein composition of a cell or organism, is growing rapidly as new technology makes it possible to analyze complex biological samples. The goal is that proteomics research will aid in the identification of diagnostic, prognostic and predictive biological markers. The majority of clinically diagnostic assays are protein based and are well adapted to standardization and clinical implementation. Furthermore, proteins are the molecules that actually change the functional state of cells, so it bears to reason that they should be assayed directly for functional analyses (Rodland 2004). Therefore, methods to discover protein expression differences could prove very useful. One popular approach to identify atypically regulated proteins is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), followed by Edman degradation or mass spectrometry to identify the proteins. 2D gel analysis allows researchers to identify differentially expressed proteins between samples. Proteomic analysis is being applied to a vast number of cancer types including breast, prostate, colon, bladder, lung, and many others. Proteomics studies provide the potential to characterize individual organelles, including the nucleus. Because the nucleus is the location of so many important biological functions, research could benefit greatly from examining this structure for differential protein expression. Identifying proteins with altered expression of specific organelles will allow us to examine their utility as biomarkers and therapeutic targets.

1.14. Urine markers for Bladder Cancer

Because of the high rate of recurrence and long term monitoring needs, a better bladder cancer diagnostic tool is needed. An optimal tool will be sensitive, specific, inexpensive, and

easy to administer and interpret. There is now a great interest in researching urine markers for bladder cancer. A few of the most prominent urine markers in the current literature will be discussed here. Table 3 provides an overview of some of the current markers and their reported specificity and sensitivity (Konety, et. al 2001). Sensitivity is defined as the number of people that have bladder cancer and were detected to have bladder cancer. Specificity is defined as the number of individuals that do not have bladder cancer in which bladder cancer is not detected.

1.14.1. NMP22

Currently two urine markers are approved for use by the FDA. NMP22 is approved by the FDA for the diagnosis of bladder cancer. NMP22, also known as nuclear mitotic apparatus protein (NuMA) is a nuclear matrix protein that is involved in the proper distribution of chromatin to daughter cells during cellular replication. NMP22 is probably released from tumor cells during apoptosis and is found at a higher concentration in individuals with bladder cancer (Saad, et. al 2002). This is an immunoassay technique that must be performed by trained technicians in a laboratory and the urine samples require stabilization for accurate test results. The sensitivity of this test has been reported to be between 68.5% and 88.5% while the specificity ranges from 65.2 to 91.3% (Dey 2004). Recently a urine-based test of NMP22 has been developed into a point of care format. The reported specificity of the point of care test is 85.7% and the sensitivity is 55.7%. While this test is convenient due to its point of care approach, it lacks the sensitivity needed for the detection of bladder cancer (Grossman, et. al 2005). NMP22 is not bladder specific, and is more useful as a marker of bladder cancer recurrence than detection.

1.14.2. BTA

The BTA test is a second generation stat test that measures human complement related H factor and has been used to detect bladder cancer using urine samples. Research has suggested that human complement factor-H may allow cells to evade the immune system and therefore confers a growth advantage to tumor cells (Saad, et al. 2002). BTA stat is a point-of-care test and can be performed and easily interpreted in a physician's office. This test is FDA approved for detecting recurrent bladder cancer. The reported sensitivity of BTA stat ranges from 62 to 77% and specificity varies from 48 to 70% (Simon, et. al 2003). This test produces a high number of false positives because it is detected in individuals with urinary tract inflammation, recent genitourinary tumors, and bladder stones. Human complement factor H is found in blood at high concentrations, so any affliction causing the presence of blood into the urine will create a positive test result (Dey 2004).

1.14.3. Survivin

Another protein currently being researched in the detection of bladder cancer is survivin, a member of the inhibitor of apoptosis protein family. Survivin inhibits apoptosis by directly inhibiting caspase-3 and caspase-7 (Ku, et. al 2004). Survivin is expressed during embryonic and fetal development, is undetectable in normal adult tissue, and is abundantly expressed in a variety of human carcinomas (Shariat, et. al 2004). Over-expression of survivin in bladder tissue is associated with aggressive disease, resistance to therapy, and poor clinical outcome in patients. Research has suggested that urinary survivin can be a useful urine biomarker to detect bladder cancer. The assay had a high specificity at 94%, however the sensitivity is only 64%. The urine

survivin assay is more sensitive than cytology when diagnosing low stage Ta tumors, but sensitivity is lower than cytology when T1 or higher tumors are examined (Shariat, et. al 2004).

1.14.4. Hyaluronic Acid and Hyaluronidase

Investigation into using hyaluronic acid (HA) and hyaluronidase (HAse) for the detection of bladder cancer is ongoing. HA is a glucosaminoglycan that is present in normal tissues and fluids, but is increased in certain tumors, including bladder (Simon, et al. 2003). Hyaluronic acid can promote tumor cell migration through cell surface receptor interactions, can open up spaces for tumor cell migration, and can form a protective coat around cells so they can escape immune surveillance. HAase is an endoglycosidase that degrades HA into small fragments that promote angiogenesis (Simon, et al. 2003). Both HA and HAase have been detected in the urine of bladder cancer patients and are both immunoassay based tests. The HA test has a reported specificity of 90% and sensitivity of 83% while the HAase test has a specificity of 81.5% and sensitivity of 83.8%. HA levels are elevated in patients irregardless of tumor grade, but HAase levels are only elevated in patients with grade 2 or 3 disease. Therefore, HA may be useful to detect bladder cancer while HAase is more useful to indicate disease grade. Combining the two assays (HA-HAase) resulted in an overall sensitivity of 92% and specificity of 84%. The combined assay has been used to explore the use of HA-HAase to monitor recurrence. When monitoring for recurrence the HA-HAase test had a sensitivity of 89% and specificity of 73%. It has also been reported that after continued follow-up, 40% of patients with initial false positive results developed recurrence disease within 3-6 months (Simon, et al. 2003). HA may not be detected in patients with high levels of HAase because this enzyme will cleave HA into small fragments that may not be detectable by the ELISA, resulting in a false negative test (Dey 2004).

1.14.5. BLCA-4

Our lab has been researching some novel nuclear matrix proteins that are specifically expressed in bladder cancer tissues. We have determined that one bladder cancer specific nuclear matrix protein, BLCA-4, is released into the urine of patients with bladder cancer and can be detected via immunoassay. An ELISA has also been developed and a large-scale clinical trial reveals that this assay can detect BLCA-4 in the urine of individuals with bladder cancer with a sensitivity of 95% and a specificity of 89%. Expression levels of this protein are not affected by hematuria, cystitis, or bladder infections as some other tests are (Van Le, et al. 2004).

Table 3: Sensitivity and specificity of methods of monitoring bladder cancer (Konety, et al. 2001)

References	No. Pts.	% Sensitivity	% Specificity
Cytology:			
Cajulis et al[7]	40	61	100
Johnston et al[25]	130	35	90
Landman et al[10]	47	47	94
Wiener et al[20]	291	59	100
Van der Poel et al[33]	138	44.8	92.5
Pode et al[9]	260	47.6	97.7
Av.		49.1	95.7
NMP-22:			
Soloway et al[15]	112	69.7	78.5
Miyanaga et al[17]	300	80.9	64.3
Landman et al[10]	47	81	77
Witjes et al[18]	50	75	81.6
Stampfer et al[19]	231	68.2	79.8
Wiener et al[20]	291	48	70
Hughes et al[21]	107	47	79
Av.		70.5	75.2
BTA:			
Sarosdy et al[23]	499	40	95.9
Ianari et al[22]	75	59	91
Johnston et al[25]	130	28	87
Landman et al[10]	47	40	73
Van der Poel et al[33]	138	34.4	81.3
Zimmerman et al[34]	22	65	40
Nasuti et al[35]	100	100	84
Av.		52.3	84.6
BTA Stat:			
Sarosdy et al[29]	220	58	72
Wiener et al[20]	291	57	68
Pode et al[32]	250	82.8	68.9
Irani et al[36]	81	65.3	71.8
Av.		65.6	65.5
BTA TRAK:			
Ellis et al[30]	216	72	75
Thomas et al[37]	220	66	69
Heicappell et al[38]	411	72	50.5
Irani et al[36]	81	77.5	62.5
Av.		71.2	64.3
FDP:			
McCabe et al[43]	242	83	66
Ewing et al[46]	210	33	84
Schmetter et al[44]	192	76	86.2
Johnston et al[25]	130	81	75
Av.		68.3	77.8
Telomerase:			
Yoshida et al[55]	109	62	96.4
Lance et al[59]	66	81	60
Landman et al[10]	47	80	80
Av.		74.3	78.8
Quanticyt:			
Wiener et al[20]	291	59	93
Witjes et al[18]	50	45.4	70.6
Av.		52.2	81.8
Fluorescence in situ hybridization, Cajulis et al[7]	40	73	100
Flow cytometry:			
Cajulis et al[7]	40	72	80
Gregoire et al[68]	166	45	87
Av.		58.5	83.5
BLCA-4, Konety et al[40]	105	96.4	100
Lewis X antigen, Pode et al[9]	260	79.8	86.4
Hyaluronic acid, Lokeshwar et al[63]	144	91.9	92.8
Hyaluronidase, Pham et al[66]	139	100	88.8

1.15. Clinical Relevance

- Over 63,000 cases of bladder cancer will be detected this year
- Current diagnostic tools are less than optimal for bladder cancer detection
- FDA approved urine markers lack sensitivity and specificity
- Early detection leads to high survival rates
- Little is known about the pathobiology of bladder cancer

1.16. Nuclear matrix

The isolation of a nuclear protein matrix was first reported by Berezney and Coffey in 1974 (Berezney, et. al 1974). The nuclear matrix is defined as an integrated three-dimensional skeletal network that organizes cellular structures and functions from the cell periphery through to the DNA (Getzenberg 1994). This structure is isolated using detergents and salts, which remove lipids, soluble proteins, intermediate filaments, DNA and RNA. The remaining framework consists of 98.2% protein, 0.1% DNA, 0.5% phospholipid, and 1.2% RNA (Berezney, et al. 1974). The nuclear matrix is the residual framework scaffolding of the nucleus and contains structural elements of the pore complexes, lamins, an internal ribonucleic protein network, and nucleoli (Replogle-Schwab, et. al 1996). The proteins that compose the nuclear matrix account for 10% of nuclear proteins (Fey, et. al 1991), and include structural proteins such as actin, intermediate keratins, tubulin, and lamins (Coffey 2002).

The nuclear lamina is a network of intermediate-sized filaments inside the nuclear membrane, made up of proteins called lamins. Lamins bind directly to DNA and also support the nuclear membrane. These proteins probably play an important role in maintaining nuclear

structure. Lamins have also been shown to associate directly with the cytoskeleton indicating that they might play a part in signal transduction between the cytoplasm and the nucleus (Bosman 1999).

The major proteins of the mammalian nuclear matrix are termed matrins. One of these proteins, termed matrin 3 appears to have a high degree of conservation among mammals, but lacks homologies with other proteins or functional motifs. Therefore little functional information is known although it does have over 40 potential phosphorylation sites suggesting that is may have a role in protein phosphorylation (Berezney, et. al 1996). Another matrin, matrin p250, has identical sequence to a hyperphosphorylated form of RNA polymerase II large subunit. This protein is believed to play a role in coordinating transcription and RNA splicing in the cell nucleus (Berezney, et al. 1996).

Previous studies have shown that a relationship exists between proteins of the nuclear matrix and proteins in the mitotic apparatus. One such protein is the nuclear mitotic apparatus protein (NuMA), or NMP22 as discussed earlier. This protein is constitutively associated with the matrix during interphase and during M phase it associates with the mitotic spindle. NuMA is thought to function in microtubule assembly, and it has been identified as a matrix attachment region suggesting an additional functional role (Mancini, et. al 1996). There still remain a multitude of nuclear matrix proteins, including autoantigens, fibronectin, keratin-like proteins, oncogene products, transcription factors, primer recognition proteins, enzymes, phospholipases, and protein kinases (Konety, et. al 1999) whose functional and structural roles still remain to be elucidated.

While the existence of the nuclear matrix is now a widely accepted hypothesis, there still exists the idea that the nuclear matrix might be an artificial result of the preparation methods

used, rather than a real *in vivo* structure. However, the data that argues in favor of the existence of the nuclear matrix as a structure is that it is observed in non-diluted nuclei through electron spectroscopic imaging, the existence of protocols to isolate this structure at physiological salt concentrations through electroelution of chromatin, the fact that chromatin loops bind to a non-chromatin network, and that functional units stay in their original place after removing chromatin and soluble proteins from the nucleus (Mika, et. al 2005).

1.16.1. Nuclear Matrix and DNA Organization

The nuclear matrix acts to maintain nuclear shape and organization, as well as functioning in many other cellular events. One of the functions of the nuclear matrix is to organize DNA. It is a great feat to pack DNA into each cell. There are about 6 feet of DNA that have to be packed into a nucleus of approximately 10 μm in diameter (Getzenberg 1994). In order to accomplish this DNA packaging, the DNA is first wrapped around histone complexes, which together form a "beads on a string" arrangement. This arrangement is then folded into a chromatin fiber, which subsequently is folded into loop domains of <20 to >200 kpb of DNA. These loops are attached by their bases to the nuclear matrix (Berezney 2002). This attachment was first visualized by releasing the supercoiled loops in the presence of ethidium bromide (Vogelstein, et. al 1980).

The sequences where the DNA attaches to the nuclear matrix have been termed matrix associated regions (MARs) or scaffold attached regions (SARs). These sequences are typically 200 bp long and are often A-T or T-G rich (Getzenberg 1994). Some proteins that interact with MARs include topoisomerase II (Berrios, et. al 1985), nuclear mitotic apparatus protein (Luderus, et. al 1994), lamins A and C (Hakes, et. al 1991), PARP-1 and PARP-2 (Tramontano,

et. al 2005), and the potential tumor suppressor CTCF (Dunn, et. al 2003). MAR binding proteins may recruit multiprotein complexes that remodel chromatin. Changing DNA organization can lead to differential gene expression, which can ultimately lead to a change in a cell's phenotype.

1.16.2. Nuclear Matrix and DNA Replication

The nuclear matrix also plays a role in DNA replication. The nuclear matrix contains fixed sites of DNA replication, which consist of the replisome complex for DNA replication (Replogle-Schwab, et al. 1996). It has also been confirmed that newly synthesized DNA is associated with the nuclear matrix. The proposed mechanism for DNA replication is that the DNA replication complexes remain bound to the nuclear matrix while DNA is reeled through (Replogle-Schwab, et al. 1996). DNA replication sites and the replication machinery are retained in an unchanged spatial distribution in nuclear matrix preparations (Nickerson 2001).

1.16.3. Nuclear Matrix and Transcription

Transcriptional regulation is another function of the nuclear matrix. 1.2% of the nuclear matrix is RNA (Berezney, et al. 1974). Most of this RNA is pre-rRNA, while small nuclear RNA complexed with proteins, and hnRNA, the precursor to messenger RNA, also associates with the nuclear matrix (Replogle-Schwab, et al. 1996). It has also been demonstrated that transcription sites and newly transcribed RNA, RNA polymerases I and II, and other transcription factors are associated with and maintain their distribution on nuclear matrix preparations (Nickerson 2001). Localization of transcription factors on the nuclear matrix suggests a role of this structure in regulating gene expression. For example, acute myelocytic

leukemia (AML) transcription factors associate with the nuclear matrix, independent of DNA binding, and this association is required for activity. A C-terminal nuclear matrix targeting signal, distinct from the nuclear localization signal was identified that is present on AML-1, AML-2, and AML-3. Such interactions provide insight as to how gene regulatory factors are targeted to the nuclear matrix. This specific targeting argues against indiscriminate attachment of proteins to the nuclear matrix. These targeting signals guide proteins to specialized domains in the nucleus to become components of functional complexes (Stein, et. al 1998). Transcription and posttranscriptional processing of gene transcripts allows selective expression of genes. There is evidence that the regulatory information for gene expression that is encoded in promoter sequences is made accessible to transcription factors by chromatin remodeling and nucleosome organization. Because chromatin attaches to the nuclear matrix, changes in nuclear shape, as occurs in cancers, affects chromatin organization and gene positioning, therefore altering gene expression (Zink 2004).

Other examples of transcription factors localizing on the nuclear matrix exist. A protein termed RFP is a protein that associates with the nuclear matrix and is involved with activation of the ret protooncogene (Getzenberg 1994). CCAAT displacement protein (CDP) and special AT-rich binding protein 1 (SATB1) are both MAR binding proteins. Both of these proteins have the ability to bind to the $gp91^{phox}$ gene, a key component of phagocyte NADPH oxidase. Both MAR binding proteins have been reported to be gene repressors of the $gp91^{phox}$ gene, demonstrating that these two nuclear matrix proteins play a role in transcriptional regulation of $gp91^{phox}$ (Fujii, et. al 2003). Another example of a nuclear matrix bound transcription factor is NMP-1. NMP-1 was initially identified as a nuclear matrix-associated DNA-biding factor that recognizes a sequence motif within a human histone H4 gene promoter. Further research led to the finding

that NMP-1 is the transcriptional regulator YY1. YY1 is a transcription factor that interacts with regulatory sequences for multiple mammalian genes. This gene has the ability to activate or repress transcription, or be functionally neutral. The presence of YY1 on the nuclear matrix indicates that this gene may function as a regulator of gene expression (Guo, et. al 1995). It has been reported that YY1 can increase the expression of the DnaJ-like heat shock protein 40 (HLJ1). HLJ1 expression is able to suppress cancer cell invasion in a lung cell line model. Therefore, the nuclear matrix associated protein YY1 may aid in regulating cancer cell invasion via transcriptional control of the HLJ1 gene (Wang, et. al 2005).

1.16.4. Nuclear Matrix and Hormones

It has also been reported that steroid receptors, including androgen, estrogen, corticosteroid, progesterone, thyroid, and vitamin D receptors, interact with the nuclear matrix (Getzenberg 1994). The interaction of steroid receptors with the nuclear matrix is steroid and tissue specific and requires the presence of an activated steroid complex bound with steroid. Hormone receptors are not found in the nuclear matrices of tissues that do not display hormone responsiveness. An example of this system is found in the rat ventral prostate. In adult male rats with intact prostates, dihydrotestosterone (DHT) receptors bind to the nuclear matrix. Following castration, androgen receptor binding to the nuclear matrix is lost. However, administration of dihydrotestosterone to the animals causes receptor binding to the nuclear matrix to return to normal (Getzenberg 1994).

The steroid receptor/ligand complex is believed to bind to the nuclear matrix via acceptor sites. An example of one of these receptor sites is RBF-1 (Leman, et. al 2002). RBF-1 is a nuclear matrix protein that has a high affinity for the progesterone receptor although its

functional relevance is not really known. Steroid receptor complexes interact with the nuclear matrix through these receptor sites, positioning themselves in the correct location for activation or repression of gene transcription (Leman, et al. 2002).

1.16.5. Nuclear Changes and Cancer

Alterations in nuclear size and shape are so common that they are often used by a pathologist to identify cancer cells. Some of the changes that can be seen in cancer cells are a change in nuclear size and shape, abnormal nucleoli, and changes in chromatin condensation (Zink 2004). Figure 2 demonstrates the appearance of a normal cell nucleus versus a cancerous cell nucleus.

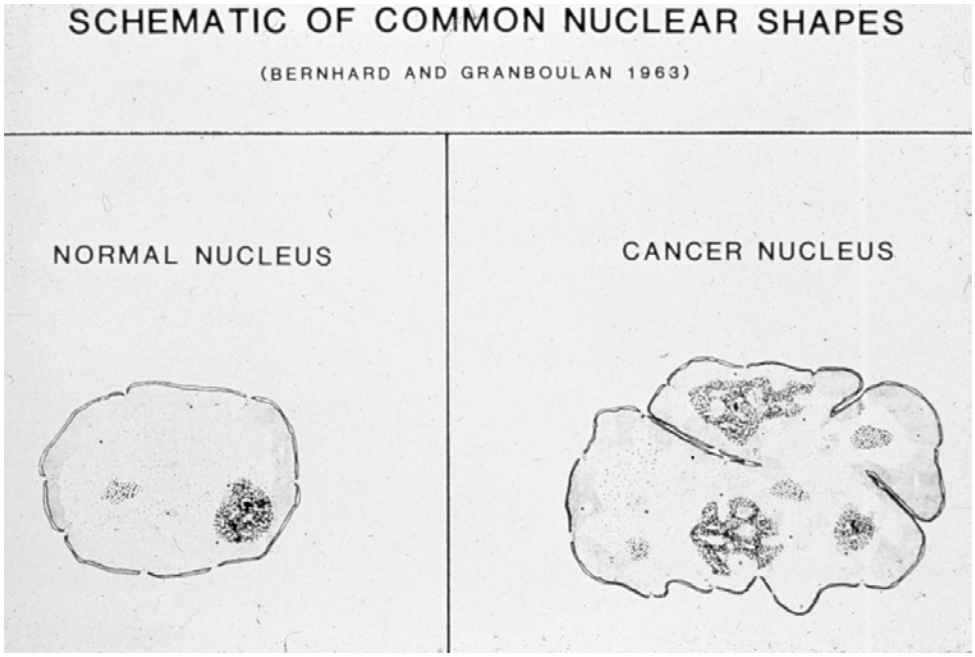

Figure 2: Morphological changes of a cancer nucleus

DNA organization and changes in nuclear shape, abnormal DNA replication, and altered transcription are all processes that have been reported to occur in cancers. Because the nuclear matrix is involved in all of these events, one can see how changes in this structure could have a large impact on the cancer process.

1.16.6. Nuclear Matrix Protein Expression (Tissue Specificity)

While many nuclear matrix proteins (NMPs) are found in a multitude of tissues and cell lines, there are some proteins, which are tissue and cell type specific. The tissue specific nuclear matrix proteins have become a popular source of research, particularly as biomarkers of disease. A PubMed search for 'nuclear matrix' results in over 3000 articles. One group has compiled a database of all the published NMPs. Currently the database contains details of 398 nuclear matrix proteins. 302 of these proteins can be matched to protein sequences in UniProt or GenBank (Mika, et al. 2005).

2D gel analysis of nuclear matrix protein extractions has revealed the presence of more than 200 nuclear matrix proteins (Zink, et. al 2004). By comparing 2D gels side by side, proteins that are tissue type or disease state specific can be identified. Nuclear matrix proteins that are specifically expressed in cancers have been identified, most notably in bladder, prostate, breast, and colon tissues (Getzenberg, et. al 1991; Getzenberg, et. al 1996; Brunagel, et. al 2002; Luftner, et. al 2002). The first nuclear matrix based test to be approved by the FDA is the NMP22 test, used to monitor recurrence in bladder cancer patients, as discussed in the urine markers section. However, its diagnostic value is diminished because it is expressed in most tissues. Getzenberg et al. have identified six bladder cancer specific proteins (Konety, et. al 2000). One of these proteins, BLCA-4, is released into the urine of individuals with bladder

cancer and an immunoassay has been developed that can differentiate between individuals with bladder cancer and those without (Van Le, et al. 2004). The discovery that nuclear matrix proteins are released into bodily fluids such as urine and serum has huge implications in cancer diagnosis. NMP based diagnostic tests for specific tumor types can be developed.

Numerous nuclear matrix proteins specifically expressed in prostate cancer have also been identified. PC-1 is a NMP found in prostate cancer tissue and not in benign hyperplastic or normal prostate tissue (Partin, et. al 1997). EPCA is a prostate specific nuclear matrix protein identified by the Getzenberg lab. Positive immunohistochemical staining of this protein was found to associate with the presence of prostatic adenocarcinoma. Furthermore, EPCA staining was identified in negative biopsies from patients later diagnosed with prostate cancer, indicating that this may be an early marker of prostate cancer (Dhir, et. al 2004). This protein has also been detected in the serum of men with prostate cancer. An immunoassay has been developed to detect this protein, which has the potential to be a diagnostic marker of this disease (Paul 2005). A TRAMP mouse model, which was developed to model the progression of prostate cancer in humans, was examined for changes in NMP expression. In total, 13 NMPs were identified that were present or absent during the progression of prostate cancer. Some proteins were present early during development of hyperplasia and were not found in late stages of the disease while other proteins appeared at the time that the mice developed neoplasia (Leman, et. al 2002). These proteins have the potential to enhance the understanding of the pathobiology and mechanism of prostate cancer as well as provide new biomarkers for the various stages of prostate cancer.

Several NMPs have been found to be specifically expressed in colon cancer tissue. Four unique NMPs were expressed in colon tumor samples but not in normal colon tissue (Brunagel,

et al. 2002). One of these proteins, CC1, has been identified as calreticulin. Calreticulin is typically associated with the endoplasmic reticulum, but a number of studies have demonstrated that this protein can localize to the nucleus. Because calreticulin expression is increased in other malignant tissues, this protein may have a limited use as a serum marker of the disease. However, it may be useful as a tissue marker of colon cancer and in making decisions regarding the malignant potential (Brunagel, et. al 2003). NMPs have also been identified that are associated with colon cancer that has metastasized to the liver. Two of these proteins, L2 and L5, are expressed in colon cancer tissue and liver metastasis tissue, but not in normal donor liver, hepatocytes, or donor colon tissue. The finding of unique NMPs in liver metastasis has the potential to elucidate the role of these proteins in metastasis as well as provide targets for therapy and early detection of metastases (Brunagel, et. al 2002).

Not only are nuclear matrix proteins expressed specifically in cancerous tissue, but normal tissue as well. Three NMPs have been identified in bladder tissue that are expressed in normal bladder tissue but not tumor tissue (Getzenberg, et al. 1996). Brunagel *et al* have also identified six NMPs that are expressed in normal colon tissue but not cancerous tissue (Brunagel, et al. 2002). These proteins have the potential to be tumor suppressors that disappear with the progression of cancer.

The altered expression of these nuclear matrix proteins is proving to be useful as biomarkers of disease, but they may also affect DNA organization, gene expression, or DNA replication. It has been reported that the bladder cancer specific nuclear matrix protein, BLCA-4, is closely related to the ETS family of transcription factors, specifically ELK3. It has also been reported that over-expression of the gene encoding BLCA-4 confers a higher growth rate in T24 cells when compared to untransfected cells or cells transfected with vector alone (Van Le, et

al. 2004). Further examination of the functional role of cancer specific NMPs may help elucidate the mechanisms of the disease.

1.17. BLCA-4

Our lab has previously identified six nuclear matrix proteins that are specifically expressed in bladder cancer when compared to normal bladder tissue. One of these proteins, BLCA-4 has been studied more extensively than the others. Protein spots isolated from 2D gels were used to obtain peptide sequence information for BLCA-4. The peptide sequences shared homology with a number of nonvertebrate proteins. However, initial peptide analysis showed no significant homology was found between BLCA-4 and any known human proteins, so we appear to have isolated a novel protein. Antibodies were produced to the BLCA-4 peptide sequence and immunoblotting was performed on bladder tissue. BLCA-4 was found to be expressed in both tumor and normal adjacent bladder tissue, but is not expressed in donor tissue. Further research was done to examine BLCA-4 expression in urine samples. After demonstrating urine-based expression by immunoblotting, an indirect immunoassay was developed. Initial results using an indirect ELISA indicated that this assay had a sensitivity of 96.4% and a specificity of 100%. Using this immunoassay we also demonstrated that levels of BLCA-4 do not vary between tumor stages or grades, and more importantly this protein is expressed in low stage tumors (Konety, et al. 2000). The effects of age, urinary tract infections, catheterization, and smoking on BLCA-4 levels were also examined. None of these variables correlated with urinary BLCA-4 levels (Konety, et al. 2000).

While these results were very promising, we wanted to produce an assay that took advantage of both the monoclonal and polyclonal antibodies we had available, as well as test this

assay using a larger patient size that included patients with benign urological conditions, bladder cancer, other types of cancer, and normal controls. A sandwich ELISA was developed that demonstrated a specificity of 95% and sensitivity of 89% (Van Le, et al. 2004).

In order to examine at what point in the disease progress BLCA-4 appears, we induced bladder cancer in a rat model. The carcinogen N-methyl-N-nitrosourea (MNU) was delivered directly into rat bladders to induce bladder cancer development. Tissue and urine samples from these rats were examined for BLCA-4 expression. BLCA-4 was identified in both tissue and urine samples collected from the rats. BLCA-4 was identified in the urine as early as 4 weeks after injection with MNU and as early as 8 weeks in the tissue (earliest time point collected). These results indicate that the appearance BLCA-4 is an early change in bladder cancer and that this protein is present before grossly visible tumors (Van Le, et al. 2004).

To try to elucidate some of the functional aspects of BLCA-4, the cDNA encoding for BLCA-4 was cloned. Analysis of the complete sequence indicates that BLCA-4 shares sequence homology with the ETS transcription factor family, with the closest similarity to the ELK-3 gene. These data indicate that BLCA-4 may be a potential transcriptional regulator. Gel shift assays were completed using a consensus ETS DNA-binding domain and indicated that BLCA-4 is able to bind to this sequence. Further information on BLCA-4 was obtained by examining if BLCA-4 could interact with specific transcription factors. The transcription factor array indicated that BLCA-4 can associate with several known transcription factors including AP-1, AP-2, NFATC, NF-E1, and NF-E2 (Van Le, et al. 2004). To further examine the function of BLCA-4, the gene that encodes this protein was transfected into a human bladder carcinoma cell line. Over-expression of BLCA-4 appeared to result in a growth advantage of this cell line (Van Le, et al. 2004).

These studies have a significant impact on the field of bladder cancer research. We demonstrated that a novel nuclear matrix protein is able to detect bladder cancer with a very high degree of specificity and sensitivity. Furthermore, we showed that this protein occurs as a very early change in bladder cancer development and may play a role in bladder cancer pathobiology. This research has given us the desire to explore additional bladder cancer specific nuclear matrix proteins.

2. Rational and Hypotheses

Nuclear matrix proteins have demonstrated the ability to be developed into biomarkers of specific cancers as well as play a role in the pathobiology of these diseases. Our lab has previously identified multiple nuclear matrix proteins that are specifically expressed in bladder cancer. One of them, BLCA-4, has been developed into a urine-based immunoassay for the detection of bladder cancer. Some of the functions of this protein have also been explored. However, there is still a great deal of functional analysis still to be examined. Exploring the role of these nuclear matrix proteins in bladder cancer will give us a better understanding of the role of these proteins in the progression of the disease as well as possible therapeutic targets to investigate.

2.1. Overall Hypothesis

Changes in structural elements of the nucleus are involved in the progression of bladder cancer and can be developed into markers of this disease

2.2. Hypothesis 1:

The nuclear matrix protein, BLCA-1, specifically expressed in bladder cancer has a role in bladder cancer pathobiology and may be developed into a marker of the disease.

The current tools used for the diagnosis of bladder cancer are less than optimal. Cytology has a poor sensitivity, is dependent on a pathologist for review, and the results are not available immediately (Al Sukhun, et al. 2003). Cystoscopy on the other hand is an invasive procedure

that is expensive and can be difficult to perform on individuals with indwelling catheters and bladder inflammation (Sengupta, et al. 2004). Because bladder cancer is a prominent disease with a high risk of recurrence, new markers must be developed. The optimal assay is specific, uses easily obtainable samples, has immediately available results, is inexpensive, and not subjective. It has already been established that nuclear matrix proteins can be developed into markers of specific diseases (Konety, et. al 1998).

Our lab has specifically demonstrated that the bladder cancer specific nuclear matrix protein BLCA-4 can be detected in the urine of individuals with bladder cancer and it appears to be a promising diagnostic tool for the detection of the disease. Because of our success developing a urine-based immunoassay with BLCA-4, we decided to explore the diagnostic potential of another bladder cancer specific nuclear matrix protein, BLCA-1.

2.3. Hypothesis 2:

The gene encoding BLCA-1 reveals novel information in regards to the function of this protein in bladder cancer pathobiology

It has previously been demonstrated that a bladder cancer specific nuclear matrix protein, BLCA-4, is related to a family of transcription factors, can bind to transcription factors, and increases growth when over-expressed in a cell line (Van Le, et al. 2004). Therefore we aimed to clone the cDNA that encodes for the BLCA-1 gene. The cDNA sequence will allow us to further explore the role of this nuclear matrix protein in the pathobiology of bladder cancer.

2.4. Hypothesis 3:

The nuclear structural protein, BLCA-4, which is specifically associated with bladder cancer plays a role in bladder cancer pathobiology

The nuclear matrix has multiple functions in a cell. It plays a role in DNA organization and stabilization, DNA replication, and transcription (Getzenberg 1994). These are all processes that have been shown to be altered in the cancer process. Therefore, changes in the nuclear matrix may have a large impact on cancer initiation and progression.

The cDNA that encodes for the nuclear matrix protein, BLCA-4, has already been cloned. This gene shares a close homology to the ETS family of transcription factors, and more specifically is closely related to the ELK3 gene. BLCA-4 has been shown to be able to interact with a number of transcription factors including AP-1, AP-2, NFATC, NF-E1, and NF-E2 (Van Le, et al. 2004). Furthermore, over-expression of BLCA-4 in a cell line demonstrated an increased growth rate in these cells. Additional functional characterization will give us more knowledge of the role of BLCA-4 in bladder cancer pathobiology.

3. METHODS

3.1. BLCA-1 Immunoassay development

3.1.1. Nuclear Matrix Preparation

Nuclear matrix proteins (NMPs) were extracted from bladder cancer tissue, tissue from normal adjacent areas of these individuals, and donor patients (individuals without urologic abnormalities who are brain dead and whose organs are being collected for transplantation) according to the method of Getzenberg *et al.*(Getzenberg, et. al 1991). Briefly, the tissue was minced into small pieces and homogenized using a polytetrafluoroethylene pestle on ice in a 0.5% solution of Triton X-100 containing 2 mM of the ribonuclease inhibitor vanadyl ribonucleoside to release proteins and lipids. The extracts were filtered through a 350 mM nylon mesh and extracted with 0.25 M ammonium sulfate to release the cytoskeletal elements. Soluble chromatin was removed using deoxyribonuclease treatment at room temperature, and the remaining fraction that contained intermediate filaments and NMPs was disassembled with 8 mM urea. The insoluble components, which mainly consisted of carbohydrates and extracellular matrix components, were pelleted by ultracentrifugation. The urea was dialyzed out and the intermediate filaments were allowed to reassemble and were subsequently removed by ultracentrifugation. All solutions contained freshly prepared 1 mM PMSF, 0.3 mM aprotinin, 1 mM leupeptin, and 1 mM pepstatin. The NMPs were then ethanol precipitated. For 2-D gel electrophoresis, the ethanol precipitated NMPs were dissolved in sample buffer consisting of 9 M urea, 65 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate,

2.2%ampholytes, and 140mM dithiorthreitol. For 1-D electrophoresis the NMPs were resuspended in 1 x PBS. The Teflon homogenization system used is not strong enough to homogenize the stromal elements of bladder, and the stromal material is filtered out so the tissue component used for analysis is urothelial. Protein concentration was quantitated by Coomassie Plus protein assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard. The final pellet containing the NMPs represented <1% of the total cellular proteins.

3.1.2. Two-Dimensional Gel Electrophoresis

High-resolution two-dimensional gel electrophoresis was performed using the Investigator 2-D gel system (Genomic Solutions, Ann Arbor, MI) as described previously (Patton, et. al 1990; Getzenberg, et al. 1991). Briefly, one hundred μg of protein was loaded per gel onto a capillary-size isoelectric focusing column. One-dimensional isoelectric focusing was carried out at 18,000 V-hr, using 1 mm X 18 inch tube gels after 1.5 hr of prefocusing. The tube gels were extruded and placed on top of 1-mm SDS Duracryl (Genomic Solutions, Ann Arbor, MI) high-tensile strength PAGE slab gels. The gels were electrophoresed at 12°C constant temperature for 4.5-5 hr. Gels were fixed with 50% methanol and 10% acetic acid. Gels were rinsed and rehydrated followed by buffering with 50mM phosphate (pH 7.2) and then treatment with 5% glutaraldehyde and 5 mM DTT. Protein molecular weight standards were Silver Standards from Diversified Biotechnology (Newton Centre, MA). Isoelectric points were determined using carbamylated standards [BDH (distributed by Gallard-Schlesinger, Carle Place, NY); and Sigma chemical Co., St. Louis, MO]. Multiple gels were run for each tissue sample and multiple tissue samples were run at different times. Only protein spots clearly and

reproducibly identical in all of the gels of a sample type were taken into account as those representing the described NMPs.

3.1.3. Protein Sequencing

Following identification of the bladder cancer-associated nuclear structural proteins, the proteins were sequenced from spots isolated by two-dimensional gel electrophoresis. The proteins were isolated according to an adaptation of a technique developed by Gevaert *et al.*(Gevaert, et. al 1995). The two-dimensional gels were negatively stained by incubation in 0.2 M imidazole for 15 min, washed several times with deionized water, and stained with warm 0.3 M zinc chloride. Deionized water was used to stop the staining, and the protein gel spots were excised and frozen at -80°C. Eight identical protein spots were then thawed, pooled, and mixed with 0.25% Coomassie Blue stain (45% methanol-9% acetic acid) for 20 min. With constant agitation, the spots were destained with destaining solution (5% methanol-7.5% acetic acid) for 1 h, washed with deionized water for 1 h, and equilibrated in sample buffer [1% SDS, 10% glycerol, 50 mM DTT, 12 mM Tris-HCl (pH 7.1)] for 1 h before being loaded into the acrylamide/agarose gel. The spots were then concentrated on a mini-agarose/acrylamide gel. The construction of the mini-agarose gel consisted of two prewarmed (60°C) glass plates (10 x 9 cm), separated by spacers 1 cm wide and 1.5 mm thick. A strip of Whatman 3-mm paper was inserted at the bottom to serve as a support for the lower agarose gel, preventing the gel from slipping during electrophoresis.

The sample well was formed by a 2-cm wide x 1.5-cm thick spacer set between two parallel spacers, each 1 cm wide x 1.5 cm thick, inserted at the center of the glass plates and attached with adhesive tape at the top edge of the back plate. The lower gel consisted of a 2-cm

deep agarose gel [1.45% agarose in 0.36 M Tris-HCl (pH 8.7), 0.1% SDS]. Once the agarose had set, it was overlayed with the polyacrylamide stacking gel [5.45% acrylamide, 0.13% bisacrylamide, 0.12 M Tris-HCl (pH 6.8), 0.1% SDS]. When the stacking gel had set, the central spacer was removed, leaving a well 2 cm high, 2 cm wide, and 1.5 mm thick. The mini-concentration gel was then mounted on a small electrophoresis tank (Bio-Rad, Hercules, CA), and the slot was filled with the equilibrated two-dimensional gel spots. The remaining volume was filled with blank gel pieces. The gels were run at 100 V, allowing the proteins to elute out of the combined gel pieces and into the acrylamide. At this time, the central spacer was reinserted into the sample well until the dye front passed the two parallel 1-cm wide spacers. At that point, the central spacer was removed, and electrophoresis continued until the dye front entered the agarose and reached the filter paper. The agarose section of the gel was fixed in fresh 50% methanol-10% acetic acid, with shaking, at room temperature for 30 min. The gel was stained with 0.05% Coomassie Blue stain (50% methanol-10% acetic acid) for 5 min and then destained in 5% methanol-7% acetic acid for 2 h with constant agitation. The protein band was then excised in a minimal volume of agarose gel, transferred into a sterile tube, and sent for peptide sequencing (Department of Biochemistry, Michigan State University, East Lansing, MI). Sequences were analyzed using the BLAST database, and sequence homologies were identified.

3.1.4. Antibody Production

The sequences obtained were used to produce peptides designed for use in antibody production (TYEEKINKQGK and WLLEGFRSRR). The peptide sequences were modified to contain a terminal cystiene for coupling purposes. The peptide sequences were verified by mass spectroscopy and conjugated to keyhole limpet hemocyanin or bovine serum albumin. Two New

Zealand White rabbits per antigen were immunized and antiserum collected. Antibodies were produced by Harlan Bioproducts for Science, Indianapolis, IN.

3.1.5. Immunoblot Analysis

Ten μg of NMP samples suspended in 1 X PBS were loaded and separated by 7.5% SDS-PAGE. Ten μl of Rainbow marker (Amersham Life Sciences, Arlington Heights, IL) were loaded in a separate lane. The proteins were transferred to a polyvinylidene difluoride membrane (Milipore, Bedford, MA) using a semi-dry transfer apparatus (Bio-Rad, Richmond, CA). The membranes were incubated overnight in PBS with 4% non-fat dry milk and 0.2% Tween-20. The membranes were incubated at room temperature with a the appropriate dilutions of antibody (Table 4) in PBS with 2% non-fat dry milk and 0.2% Tween-20 for 1 hr. Antibody incubation was followed by three 15 minute washes with 1 X PBS and 0.2% Tween-20. Secondary antibody was incubated at room temperature for 1 hr. The membrane was washed again with PBS and 0.2% Tween-20. Detection was achieved using a chemiluminescence reaction using the ECL Immunoblot kit (Amersham Life Sciences).

Table 4: Antibodies used in immunoblots

Antibody	Species	Source	Cat #	Concentration	Secondary Ab concentration
BLCA-1	Rabbit	Harlan	N/A	1:50	1:20000
BLCA-4	Rabbit	Biosource	N/A	1:100	1:20000
IL1-α	Mouse	Santa Cruz Biotech	Sc-9983	1:50	1:10000
IL-8	Mouse	Santa Cruz Biotech	Sc-8427	1:50	1:10000
Thrombomodulin	Mouse	Santa Cruz Biotech	Sc-13164	1:100	1:10000
Actin	Goat	Santa Cruz Biotech	Sc-1615	1:100	1:10000

3.1.6. Enzyme Linked Immunosorbant Assay (ELISA)

Urine samples were obtained from 25 patients with active bladder cancer at the time of collection, 18 normal individuals who did not have any urological abnormalities, 8 individuals with spinal cord injuries, 10 males with prostate cancer, and 10 patients with kidney cancer under IRB approved protocols. The cancer patients ages ranged from 41-86 and normal individuals ranged from 22-57. Seven of the normal donor patients were males and 11 were females, six of the spinal cord patients were males and two were females, and eight of the kidney cancer patients were males and two were female. Eighteen of the bladder cancer patients were males, four were females, and three were unknown. Three of the bladder tumor samples were grade 1, nine were grade 2, and eleven were grade 3. Pathology reports were not available for two of the patients. While we were able to obtain the data for tumor grade on these patients, stage information was only available on a fraction of the samples. One hundred µl of urine or serum was loaded into a 96-well flat bottom plate and incubated overnight at room temperature. 0.5 µg Rabbit IgG and 1 ng BLCA-1 peptide was used as a positive control. The following day, the plates were rinsed with deionized water and blocked with bovine serum blocking agent for 30 min. The plates were rinsed again and incubated for 2 hr with anti-BLCA-1 antibody. Rabbit pre-immune serum was used as a negative control. Following three washes with deionized water, secondary antibody, goat-anti-rabbit conjugated with alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD), at a dilution of 1:5,000 was incubated for 2 hrs at room temperature. Detection was accomplished using TMB Microwell Peroxidase Substrate (1-Component) (Kirkegaard & Perry Laboratories). Plates were read at 630 nm.

3.2. BLCA-1 cloning

3.2.1. Polymerase Chain Reaction

One μg RNA isolated from bladder tumor tissue (RNeasy® midi kit, Qiagen, Valencia, CA) was reverse transcribed to cDNA using the Reflection RT kit (Active Motif, Carlsbad, CA). PCR amplifications were performed using 2 μg cDNA, 10 μM forward and reverse primer, water up to 25 μL, and 25 μL RedMix Plus 1.5mM $MgCl_2$ (Gene Choice, Frederick, MD). PCR was done using the following conditions: 94° for 1 min, denaturation at 94° for 30 seconds, annealing at 52° for 30 seconds, and extension at 68° for 1 minute for a total of 30 cycles. Degenerate primers were made to the peptide sequences obtained from Edman degradation sequencing. Combinations of forward and reverse primers were tried until a band was amplified. Degenerate primers that produced a band and were used for subsequent cloning reactions consisted of:

BLCA-1 forward primer: 5'-TACTTYAARCTYTACCTYGTYATRCANCTY-3'

BLCA-1 reverse primer: 5'-YCTYCTRCTYCTRAANCCYTCNAGNAGCCA-3' (Invitrogen, Carlsbad, CA).

3.2.2. DNA Gel Extraction

DNA was extracted from a 1% agarose gel using the QIAquick® Gel Extraction Kit protocol (Qiagen, Valencia, CA) as per manufacturer's instructions. In summary, following PCR, four bands were cut from a 1% agarose gel and pooled together. The agarose gel was completely dissolved in buffer, mixed with isopropanol, and added to the QIAquick® column. Following centrifugation the column was washed with the appropriate buffer. Elution was completed using 30 μl deionized water. A 260/280 reading was performed to confirm purity and DNA concentration.

3.2.3. Cloning Reaction

Cloning was performed using the TOPO® TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Four µl of purified DNA was added to 1 µl salt solution, and 1 µl TOPO® vector. The control reaction was performed using 1 µl salt solution, 4 µl sterile water, and 1 µl TOPO® vector. The reactions were incubated for 5 minutes at room temperature and then placed on ice.

3.2.4. Transformation

Transformation of One Shot® TOP10 Competent Cells was performed using the TOPO® TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA), following the chemical transformation protocol. Briefly, 2 µl of the TOPO® cloning reaction was added to a vial of One Shot® chemically competent *E. coli* and incubated on ice for 5 minutes. The cells were heat shocked for 30 seconds at 42°C and transferred to ice. 250 µl of S.O.C. medium (provided with kit) was added and tubes were shaken horizontally at 37°C for 1 hour. 50 µl of each transformation were spread on prewarmed LB/amp plates coated with X-gal and incubated overnight at 37°C.

3.2.5. Plasmid Purification

Transformed colonies were picked and grown overnight in 4 mL LB broth containing 100 µg/mL ampicillin. The plasmid was purified using the QIAprep® Miniprep kit (Qiagen, Valencia, CA). Pelleted bacterial cells were resuspended in the appropriate buffers. The samples were centrifuged and supernatants applied to the QIAprep spin columns. The columns

were washed and the DNA was eluted with 50 µl water. DNA purity and concentration was examined by a 260/280 reading.

3.2.6. Restriction Digest

Proper insert of BLCA-1 into the plasmid was examined by a restriction digest. 10 µl of plasmid DNA was added to 7 µl water, 2 µl EcoR1 buffer and 1 µl EcoR1 enzyme. Following incubation at 37° for 2 ½ hours the product was run on a 1% agarose gel. Twelve µl of plasmid DNA containing the proper insert was sent for sequencing (Johns Hopkins University, Baltimore, MD).

3.3. BLCA-4 functional analysis

3.3.1. Transfection of BLCA-4

BLCA-4 cDNA was transfected into HUC, a non-tumorigenic human uroepithelial cell line transformed with SV40, (American Type Culture Collection), using the Lipofectamine Plus kit (Invitrogen, Carlsbad, CA). HUC cells were plated to ~70% confluency in six well plates. Each well of a six well plate was transfected with increasing amounts of DNA (0.5, 1, 2, 3, or 4 µg BLCA-4 cDNA or 1 µg vector only). Cells were selected with zeocin 48 hours after transfection in order to create stable transfectants. Each clone was isolated using clonal discs and transferred to a 24- well plate. Once confluent, the transfected cells were gradually transferred to a six-well, then T25 cm^2 and subsequently T75 cm^2 flasks to isolate cell lysates or RNA.

3.3.2. Total Cell Lysate Isolation

Cell lysates were isolated from confluent T75 flasks as described previously (Leman, et. al 2003). Briefly, whole cell extracts were prepared by lysing the cells in extraction buffer (20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM $Na_3P_2O_7$, 1 mM Na_3VO_4, 1 mM ethylenediaminetetraacetic acid, 1 mM egtazic acid, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 420 mM NaCl, 20% glycerol, 1 µg/ml leupeptin and 1 µg/ml aprotinin), followed by snap freezing. The supernatant was then centrifuged and harvested. Protein concentrations were determined using a Coomassie plus (Pierce, Rockford, Illinois) protein assay kit according to the manufacturer protocol.

3.3.3. RNA Isolation from Cells

RNA was isolated from cells using the RNeasy midi kit (Qiagen, Valencia, CA). Briefly, cells from two T75s were pelleted and resuspended in lysis buffer. Homogenization was accomplished by passing the cells through an 18 gauge needle, followed by centrifugation. Supernatants were mixed with one volume ethanol and then added to the column provided. Following centrifugation the column was washed and elution was accomplished using 150 µl RNase-free water.

3.3.4. Cell proliferation assay

Two thousand cells of each clone were plated in 8 wells of a 96 well plate on day 1. MTT assays were performed on days 3, 4, 5, and 6. The assay was performed by making 5 µg/µL of Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich, St. Louis, MO) in 1X PBS. 50 µL of MTT solution was added to each well and incubated for 3 hours. The MTT solution was

aspirated off and 200 μL of Dimethyl Sulfoxide was added to each well. The absorbance was read immediately at 595 nm.

3.3.5. Polymerase Chain Reaction

One μg RNA isolated from transfected clones was reverse transcribed to cDNA using the Reflection RT kit (Active Motif, Carlsbad, CA). PCR amplifications were performed using 2 μg cDNA, 10 μM forward and reverse primer, water up to 25 μL, and 25 μL RedMix Plus 1.5mM MgCl2 (Gene Choice, Frederick, MD). PCR was done using the following conditions: 94° for 3 min, denaturation at 92° for 30 seconds, annealing at 58° for 30 seconds, and extension at 72° for 2 minutes 30 seconds for a total of 30 cycles. Degenerate primers consisted of

BLCA-4 forward primer: 5'-CCNGCRTTYAAYTGRCTDATYTC-3'

BLCA-4 reverse primer: 5'-GTNTAYGARGAYATHATGCARAA-3' (Invitrogen, Carlsbad, CA).

3.3.6. Microarray analysis

Total RNA samples were biotin-labeled using the GeneChip® Expression 3' Amplification One-Cycle Target Labeling and Control Reagents Kit as suggested by the manufacturer. Briefly, 1 μg of total RNA in 8 μL Nuclease-free water were spiked with poly-A RNA controls (Affymetrix, Santa Clara, CA) and then incubated with 2 μL of 50 μM T7-Oligo $(dT)_{24}$ Primer (Affymetrix, Santa Clara, CA) at 70^{o}C for 10 minutes and cooled on ice. First-strand cDNA was synthesized by reverse transcription and second-strand cDNA was synthesized with E. coli DNA polymerase I and RNase H and T4 DNA polymerase. Double-stranded cDNA was purified using the Sample Cleanup Module (Affymetrix, Santa Clara, CA). Purified double-stranded cDNA was

then used in the *in vitro* transcription reaction at 37 ^{o}C for 16 hours in a 40 μL reaction volume, containing purified ds-cDNA, 4 μL of 10X IVT labeling buffer, 12 μL IVT labeling NTP mix, 4 μL IVT labeling enzyme mix and variable amount of RNase-free water. One μL of purified biotin labeled cRNA was then analyzed for purity and concentration by ND-1000 Spectrophotometer and Agilent 2100 Bioanalyzer. 15 μg of purified cRNA was incubated with fragmentation buffer (Affymetrix, Santa Clara, CA) at 94 ^{o}C for 35 minutes. 1 μL aliquot was used to assess complete fragmentation in the Agilent Bioanalyzer. 15 μg of fragmented cRNA sample was hybridized to Affymetrix GeneChip HG-U133 Plus 2.0 arrays. Each array was then washed, stained with streptavidin-phycoerythrin in a GeneChip® Fluidics Station 400 and scanned by a GeneChip® Scanner 3000 (Affymetrix, Santa Clara, CA) as recommended by the manufacturer. Quality Control (QC) parameters and numerical gene expression data were derived from the MAS 5.0 algorithm of the GCOS software (version 1.1, Affymetrix, Santa Clara, CA). Microarray analysis was performed by the University of Pittsburgh Medical Center Department of Pathology array facility. In order to choose genes of interest for further analysis, a cutoff of five-fold increase or decrease was chosen. It is acknowledged that this artificially defined cutoff will miss important changes of lower magnitude but this point was chosen to examine gross changes in expression.

4. Proteomic Analysis of Bladder Cancer

4.1. Introduction

Nuclear structural alterations are characteristic of neoplastic transformation. Current methods for the diagnosis of bladder cancer involve examining the nuclear morphology of cells in urine samples. Proteomic analysis has become a large area of research in the past few years. Proteomics is defined as the study of the proteomes of tissues and body fluids (Lee, et. al 2005). Proteomic analysis often involves comparison of different states such as cancerous and normal tissue. One of the most commonly used techniques involves separating proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). This procedure separates proteins first by their electrical charge and then by their molecular weight. Protein expression profiling of bladder cancer tissue has been explored. 2D analysis of normal or low-grade transitional cell carcinomas has lead to the identification of four proteins which are present in these samples and are not in high-grade TCCs (Lee, et al. 2005), demonstrating the ability of this procedure to identify markers which are selectively expressed in various disease states. These proteins can then be isolated and further characterized.

The nuclear matrix is the skeletal network that organizes cellular structures and functions. Some of the functions of the nuclear matrix include DNA organization and stabilization, DNA replication, and synthesizing RNA (Getzenberg 1994), which all have the potential to be altered during the progression of cancer. Research has demonstrated that the nuclear matrix protein

composition can be altered during malignant transformation and can be used as markers for prostate and breast cancer phenotypes (Konety, et al. 1998). Based on these studies we wanted to examine changes in nuclear matrix protein composition of normal bladder tissue compared with cancerous bladder tissue.

4.2. Results

After two dimensional gel electrophoresis of nuclear matrix proteins extracted from bladder tumor tissue and normal adjacent tissue, six proteins were identified that are specifically expressed in the tumor tissue (Figure 3B), while three proteins were identified that are expressed only in normal adjacent tissue (Figure 3A).

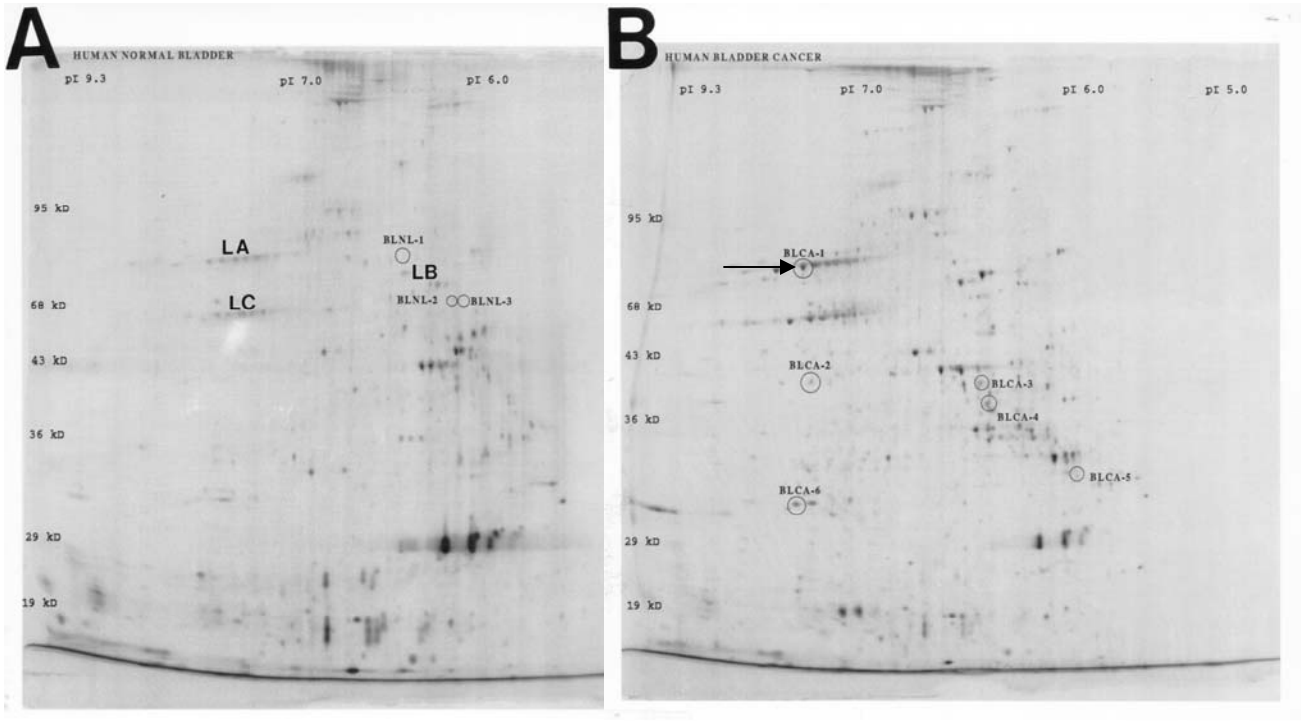

Figure 3: Silver stained two-dimensional gel electrophoresis of bladder nuclear matrix proteins

2D gel analysis of normal adjacent bladder tissue (A) and bladder cancer tissue (B). Six proteins have been identified that are bladder cancer specific. BLCA-1 is indicated by the arrow at 72kD with an isoelectric point of 7.7.

Spots corresponding to BLCA-1, BLCA-2, and BLCA-6 were isolated and concentrated separately to obtain peptide sequence data corresponding to these proteins. The peptide sequences as well as their BLAST matches, organism of origin, and amount of homology are described in Tables 5, 6, and 7. According to an extensive analysis of the sequence data, it appears that all three are novel proteins. While we believe that the peptides derived from each of these spots results from a single protein, this has not been definitively proven. Some of the experiments proposed in the future directions section of this thesis will examine this more thoroughly. Although homology of the peptides to known proteins suggests that each may contain potentially interesting motifs, little functional information is currently available. None of the human protein matches found from BLAST analysis have the same molecular weight or isoelectric point of BLCA-1, BLCA-2, or BLCA-6. Based on antigenic potential and interesting homology to blast sequences, the BLCA-1 peptides TYEEKINKQGK and WLLEGFRSRR, were conjugated to carrier proteins for antibody production. The BLCA-2 peptide sequence ERLENENVTE was used for antibody production, and the BLCA-6 peptide sequences QIEATIN and QSFTMVADTPENL were conjugated to carrier proteins for antibody production.

Table 5: Sequence data obtained from BLCA-1 spots isolated by two-dimensional gel electrophoresis

Sequence Results	Database Matches	Organism	Homology
(N)XLDQEVNT(E)	similar to 60s ribosomal protein L17 choline kinase GmCK2p-like protein glucose-1-phosphate adenylyltransferase	Rattus norvegicus Arabidopsis thalaiana Haemophilus influenzae	7/8 7/10 6/7
ALILELEIEN	mitochondrial processing peptidase-beta	Homo sapiens	7/7
MKFEMEQYL(E)	RNA1 polyprotein keratin-9 ovariam tumor protein isoform cag island protein	Tobacco ringspot virus Homo sapiens Drosophila melanogaster Helicobacter pylori	8/10 7/8 6/8 6/10
TYEEKINKQGK	phosphoribosylformylglycinamidine synthetase	Salmonella typhimurium	6/7
WLLEGFRSRR	cell division protein ftsJ similar to G protein-coupled receptor MRGX3 RNA polymerase I transcription factor RRN3	Rickettsia prowazekii Homo sapiens Homo sapiens	7/7 8/10 7/8

Table 6: Sequence data obtained from BLCA-2 spots isolated by two-dimensional gel electrophoresis

Sequence Results	Database Matches	Organism	Homology
IKEVYMHALY	glutamate-ammonia ligase	Cow	7/9
	glycoprotein B	Human herpesvirus 6	6/6
	similar to exonuclease	Rattus norvegicus	6/6
	valyl-tRNA synthetase	Danio rerio	6/7
	insulin degrading enzyme	Rattus norvegicus	6/9
	flagellar protein	Xanthomonas campestris	5/6
ERLENENVTE	rust resistance-like protein	Zea mays	7/9
	transcriptional regulatory protein, AsnC family	Archaeoglobus fulgidus	8/10
	transposase	Nostoc sp.	7/7
	Tropomyosin 2	Schistosoma mansoni	7/10
	oxidosqualene cyclase	Allium macrostemon	6/7
	PI3-kinase	Dictyostelium discoideum	
	cell division protein (mukB)	Haemophilus influenzae	
	cardiac muscle factor 1	Chicken	
XXVEANVQ	acetyl CoA carboxylase	Zea mays	6/6
	phosphoinositide-3-kinase	Homo sapiens	5/6
	Titin	Rabbit	5/6

Table 7: Sequence data obtained from BLCA-6 spots isolated by two-dimensional gel electrophoresis

Sequence Results	Database Matches	Organism	Homology
QIEATIN	putative replication protein A1	Arabidopsis thaliana	6/6
	ferrichrome ABC transporter	Bacillus halodruans	6/6
	2-isopropylmaltate synthase	Buchnera aphidicola	6/7
	intracellular proteinase inhibitor	Bacillus halodruans	6/6
	Tektin 2	Mus musculus	6/7
YKQQLELVKQV	DNA polymerase I -3'-5' exonuclease	Thermoanaerobacter tengcongensis	7/11
	Tryptophan synthase alpha chain	Cyanidium caldarium	7/8
	xanthine guanine phosphoribosyl transferase	Helicobacter pylori	7/7
EKFVYENALK	putative pre-mRNA splicing factor	Oryza sativa	7/7
	myozenin I	Homo sapiens	6/7
	cellulose biosynthesis protein	Agrobacterium tumefaciens	7/8
	merozoite surface protein 1, precursor	Plasmodium falciparum	8/10
QSFTMVADTPENL	LIM and SH3 protein 1	Homo sapiens	13/13
	Similar to nebulin	Norway Rat	8/11
EKYVYMHALK	cGMP dependent protein kinase	Drosophila	6/6
	histone deacetylase	Plasmodium falciparum	6/6
	Lipase chaperone	Acinetobacter lwoffii	7/8
	similar to exonuclease	Norway rat	6/6
	replicase	Bovine coronavirus	6/8
	envelope protein	Bovine foamy virus	6/8

5. Utilization of the Novel Marker, BLCA-1, for the Detection of Bladder Cancer

5.1. Introduction

Bladder cancer is one of the most frequently diagnosed cancers and is a significant source of morbidity and mortality throughout the world. According to the American Cancer Society (2005), in the United States, bladder cancer is the 4th most commonly diagnosed cancer in males and the 10th most commonly diagnosed in women, accounting for over 2% of all cancer deaths. More than 90% of bladder cancers are transitional cell carcinomas (Al Sukhun, et al. 2003). At the time of diagnosis, about 75% of patients have superficial bladder cancer, with tumors confined to the mucosa or lamina propria. Superficial bladder cancer has a very high rate of recurrence with up to 70% of patients suffering from recurrence of this disease, and 10% to 30% will present with grade and stage progression (Kwak, et al. 2004).

Bladder cancer presents with few symptoms in which to aid in the detection of this cancer. The most common symptom that is seen in ~80% of patients, is the presence of blood in the urine, or hematuria. However, most people that exhibit hematuria, up to 90%, do not have bladder cancer. Some other symptoms are painful or difficult urination, increased frequency of urination, or abdominal pain (Van Le, et al. 2004), but these symptoms are also indicative of diseases other than bladder cancer. The most common risk factors for bladder cancer are exposure to the chemical carcinogens arylamines and smoking. These two risk factors alone are thought to contribute to over 50% of all bladder cancer cases (Moyad 2003).

Because of the high diagnosis rate and lack of symptoms for this disease, a sensitive and specific detection tool that can be easily sampled is needed. The current standard for detection of bladder cancer relies on cystoscopy and cytology. Cytology involves microscopic evaluation of cells in the urine from patients. This test has a high specificity, but lacks sensitivity in detection of low grade tumors, as well as requires a trained pathologist for review (Al Sukhun, et al. 2003).

Two urine-based tests that are commercially available are NMP22 and BTA. The sensitivity of the NMP22 test has been reported to be between 68.5% and 88.5% while the specificity ranges from 65.2 to 91.3% (Dey 2004). The reported sensitivity of the BTA stat test ranges from 57 to 83% and specificity varies from 68 to 72%. Another protein currently being researched for the detection of bladder cancer, survivin, has a high potential specificity at 94%, however the sensitivity is only 64% (Shariat, et al. 2004). Therefore, the use of these urine markers in the detection of bladder cancer is limited by their somewhat poor sensitivity and specificity.

Our lab previously identified six nuclear structural proteins (BLCA1-6) that are specifically expressed in bladder cancer tissue (Getzenberg, et al. 1996; Konety, et al. 2000). The nuclear matrix is the support scaffold of the cell nucleus. This structure has a variety of functions, many of which have implications in cancer progression. The nuclear matrix plays a role in determining nuclear morphology, organizing DNA, stabilizing and orienting DNA during replication, organizing gene regulatory complexes, and synthesizing RNA (Konety, et al. 1999).

One of the bladder cancer specific nuclear proteins, BLCA-4 has previously been sequenced, and specific antibodies have been produced. It has been demonstrated via one-dimensional gel electrophoresis that this protein is expressed in bladder cancer tumor tissue as

well as normal adjacent tissue, but is not expressed in tissue from normal donor patients. An indirect ELISA has also been developed that can detect this protein in the urine of individuals with bladder cancer with a sensitivity of 96.4% and a specificity of 100% (Konety, et al. 2000). The cDNA has been cloned for this protein and some of the functional aspects of this protein have been examined. This protein appears to be a novel member of the ETS transcription factor family of proteins, sharing the closest similarity with the ELK3 gene (Van Le, et al. 2004).

The focus of this study is to examine the expression pattern of another of the bladder cancer specific nuclear structural proteins, BLCA-1. In order to examine this protein, we have isolated it from two dimensional gels and obtained sequence data for the protein. This data was used to produce antibodies which have been used to examine the expression of BLCA-1 in bladder tissue and urine via western blots and ELISAs. The results indicate that BLCA-1 can be specifically detected in the tissue and urine of individuals with bladder cancer and can be utilized as an assay to detect this disease. Furthermore, BLCA-1 can be detected in serum samples from individuals with bladder cancer and may associate with stage of disease.

5.2. Hypothesis

The nuclear matrix protein, BLCA-1, specifically expressed in bladder cancer has a role in bladder pathobiology and may be developed into a marker of the disease.

5.3. Results

The expression of BLCA-1, as evaluated by the anti-BLCA-1 antibodies, was first tested via immunoblot using nuclear matrix proteins extracted from human donor bladder tissue (tissue from individuals with no urologic abnormalities, normal adjacent bladder tissue (tissue from normal areas adjacent to a bladder tumor), or bladder tumor tissue. Both antibodies show the same specificity in bladder tissue (Figure 4). However, the antibody produced against the sequence WLLEGFRSRR had lower background so all remaining experiments were carried out using this antibody. The BLCA-1 antibodies recognize their peptide sequence as demonstrated by immunoblotting using 1μg of peptide in PBS (data not shown).

BLCA-1 (72kD) is not recognized in normal adjacent tissue, or tissue from normal organ donors, but is detected in bladder tumor tissue at varying levels (Figure 4). A higher band is seen in the tumor samples that is believed to be a non-specific background band. The primary antibody was not preabsorbed with peptide in order to examine specificity but could be applied in future experiments. This expression pattern does not appear to be related to age, as the majority of the donor tissues tested were from individuals ranging in age from 53-69, which is similar to the age range for the bladder cancer patients. A band of ~50kD is present in the normal and donor samples, but is a background band that is often seen when using anti-NMP antibodies on nuclear matrix preparations. Immunohistochemical staining of bladder tissue was attempted to provide additional confirmation of tumor specificity, but the antibodies were not conducive to this type of assay.

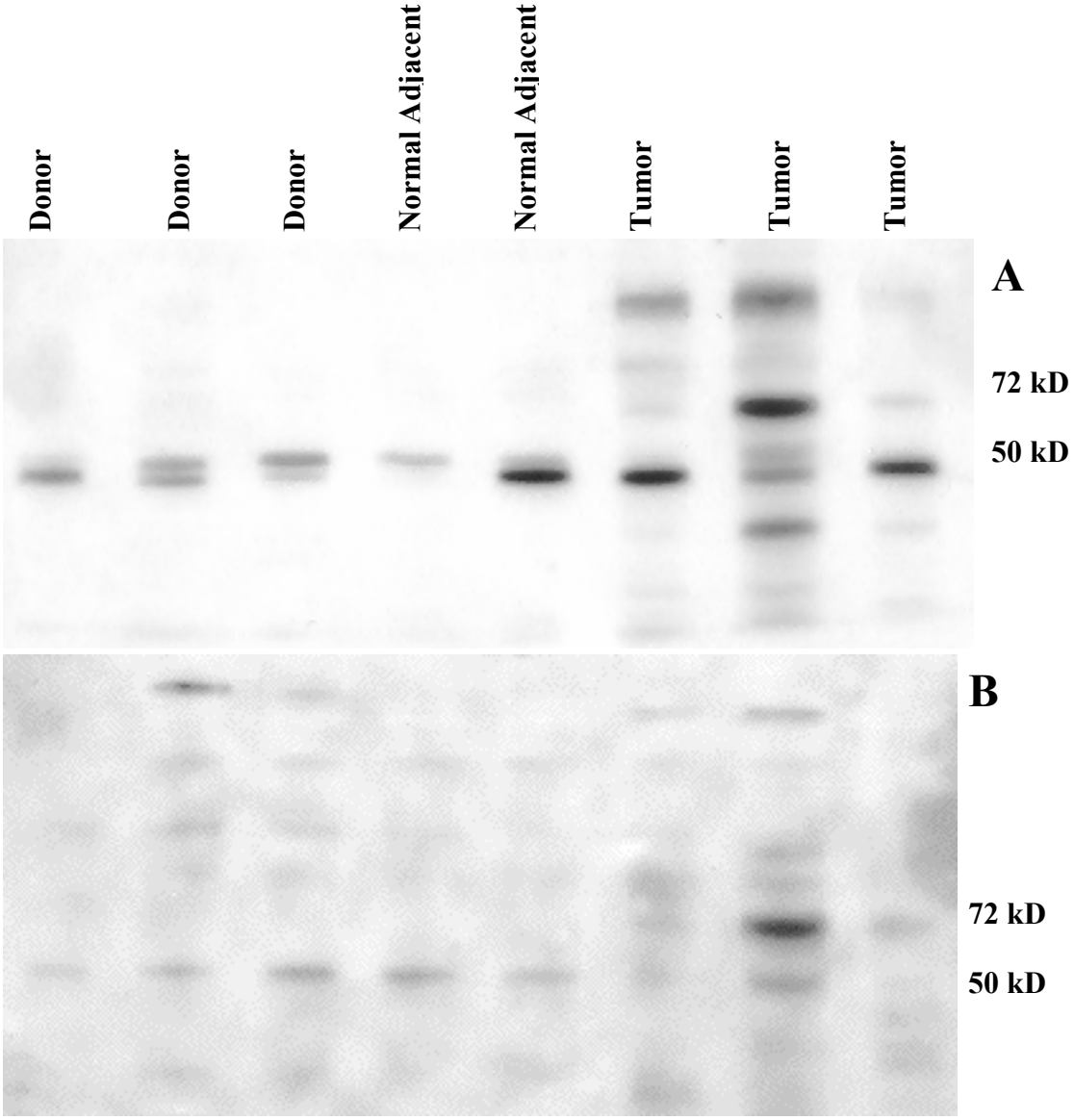

Figure 4: Immunoblot of BLCA-1 in bladder tissue

Anti-BLCA-1 antibodies were used to detect the protein in bladder cancer tissue samples. A. Immunoblot using the antibody to peptide sequence WLLEGFRSRR. B. Immunoblot using the antibody to peptide sequence TYEEKINKQGK. The antibodies do not react with any of the normal adjacent bladder tissue or bladder tissue from donor patients without urologic malignancies. The band of ~50kD is a background band that is often seen when probing with anti-NMP antibodies.

BLCA-1 localizes to the nucleus as demonstrated by cellular fractionation of the T24 bladder cancer cell line (Figure 5).

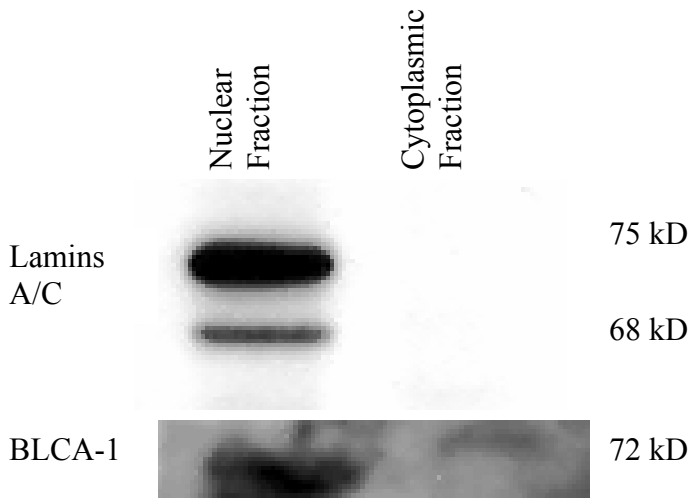

Figure 5: Immunoassay of BLCA-1 localization

An anti-BLCA-1 antibody detects BLCA-1 in the nuclear fraction of cells but not in the cytoplasmic fraction.

To further test the specificity of this antibody, nuclear matrix proteins from other tissue types were tested by immunoblot. BLCA-1 is not detected in prostate tumor tissue, colon tumor tissue, or normal organ donor tissue (Fig. 6). BLCA-1 was identified in extracts from renal tumor tissue. Detection of BLCA-1 in tissue from renal cell carcinoma may be observed because kidney and bladder cancers are both urogential cancers. Bladder cancer often behaves as a field change disease in which the entire urothelium from the renal pelvis to the urethra is susceptible to malignant transformation (Campbell, et al. 2002) and may explain why we detect BLCA-1 in the tissue of patients with renal cancer. The 50 kD band that we often detect with nuclear matrix antibodies is not reproducibly seen in every tissue type. It is detected in nuclear matrix proteins from bladder tissue, renal tissue, and colon tissue. However, it is not detected in prostate tissue in this blot, although our lab has previously detected this band in prostate nuclear matrix preparations.

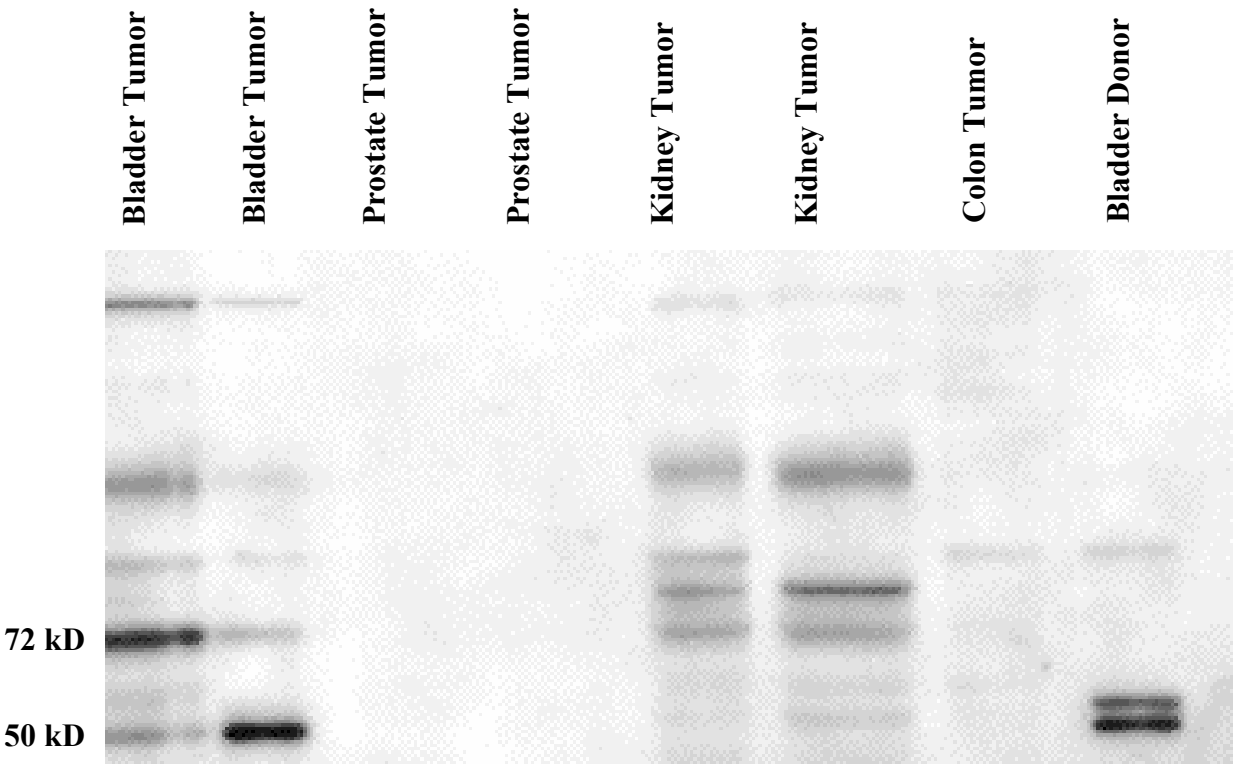

Figure 6: Immunoblot to detect BLCA-1 in various tumor types

BLCA-1 is detected in bladder tumor tissue as well as kidney carcinoma tissue, but not in prostate tumor tissue, colon tumor tissue or bladder donor tissue.

Although the tissue staining patterns support the use of this marker, the goal of these studies was to determine if the marker could be detected in the urine. Utilizing the anti-BLCA-1 antibody, the protein is detected in the urine from individuals that have been diagnosed with bladder cancer, but not in the urine of normal individuals (Figure 7). The protein appears to be slightly smaller in the urine than in the tissue, perhaps due to proteolytic cleavage in the urine.

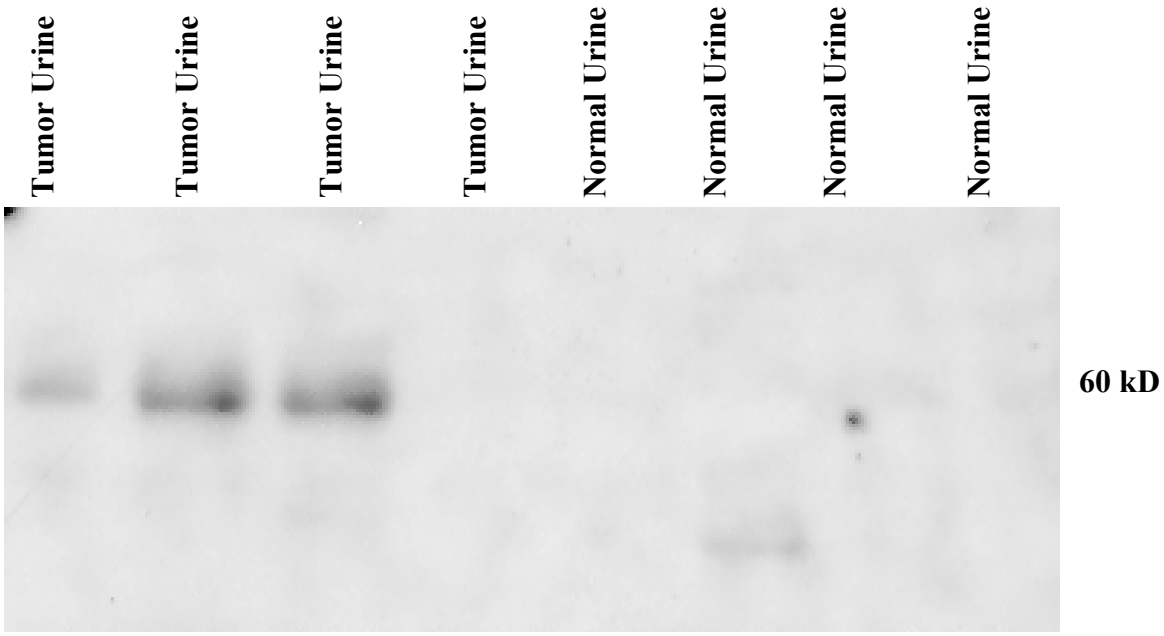

Figure 7: One dimensional immunoblot of voided urine samples

BLCA-1 is able to be detected in the voided urine of patients with bladder tumors but is not detected in the urine of normal individuals.

For clinical applications, we developed an ELISA that detects BLCA-1 specifically in the urine of bladder cancer patients. The ELISA detects small amounts (1ng) of peptide prepared in a solution of PBS. A training set consisting of eight urines from normal individuals and eight patients with bladder cancer was used to define a cutoff for the assay. The mean absorbance was 0.017 with a median value of 0.014 for normal individuals and mean of 0.052 with a median value of 0.036 for individuals with bladder cancer. Based on these initial results, a cutoff absorbance value of 0.025 O.D. units was defined, with values less than 0.025 O.D. being considered normal and higher values indicating bladder cancer. The previously defined cutoff was applied to subsequent tests on the samples listed in Table 8.

Table 8: Patient demographics

	Sample Number	Gender		
		M	F	unknown
Bladder cancer	25	18	4	3
Normal (no urological abnormalities)	18	7	11	
Spinal cord injuries	8	6	2	
Kidney cancer	10	8	2	
Prostate cancer	10	10		

Spinal cord injury patients present a unique population in which to use in this assay because they have a high risk of bladder cancer and other urinary conditions such as chronic catheterization and cystitis (West, et. al 1999), (Bejany, et. al 1987). This urine-based immunoassay is able to detect BLCA-1 levels above the cutoff in 20 out of 25 urine samples from patients with clinically diagnosed bladder cancer giving a sensitivity of 80% and only detected protein levels above the cutoff in 6 out of 46 samples from individuals without bladder cancer, resulting in a specificity of 87% (Figure 8). The spinal cord injury sample that is above the cutoff, is from a patient diagnosed with cystitis. However, another sample from a patient with cystitis is below the cutoff, so more samples from patients with cystitis need to be collected to determine whether an association exists between cystitis and BLCA-1 levels. BLCA-1 is stable when stored at -80° and can be detected in the urine up to three years after storage at these conditions.

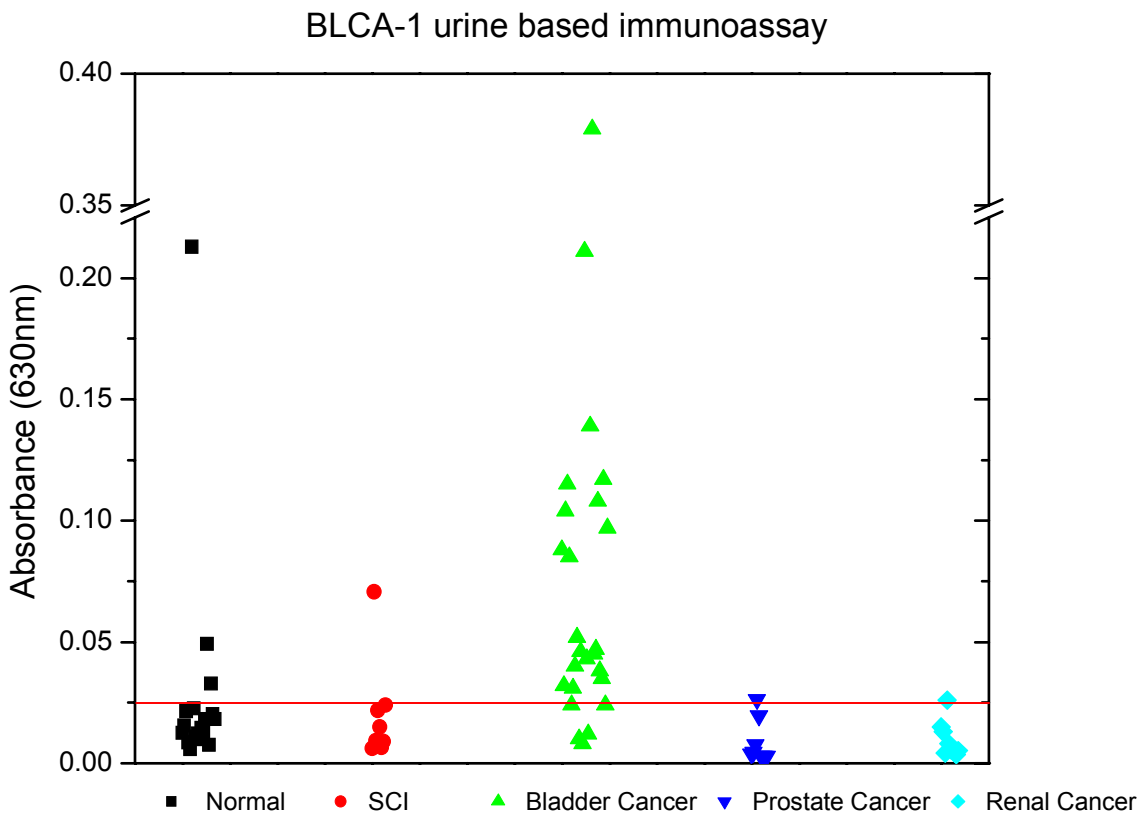

Figure 8: BLCA-1 urine-based immunoassay

BLCA-1 is detectable in the urine of bladder cancer patients compared to normal patients, patients with spinal cord injuries, patients with prostate cancer, or patients with kidney cancer via immunoassay.

The BLCA-1 protein levels in bladder cancer patients are statistically significantly higher than the levels in normal individuals (p=0.007), those with spinal cord injuries (p=0.001), individuals with prostate cancer (p=1.0E-04), or patients with kidney cancer (p=1.1E-04), using the Wilcoxon rank-sum test (Table 9).

Table 9: Average and median O.D. values, standard deviation, and p-values for each patient group tested

	Average (O.D. units)	Median (O.D. units)	Std Dev	p-value vs bladder cancer
Normal	0.028	0.015	± 0.047	0.007
SCI	0.020	0.012	± 0.021	0.001
Prostate Cancer	0.009	0.004	± 0.008	1.0E-04
Kidney cancer	0.009	0.006	± 0.007	1.1E-04
Bladder Cancer	0.078	0.047	± 0.079	

The sensitivity and specificity of the assay across various ranges of BLCA-1 measurements are shown in a ROC curve (Figure 9). A ROC curve is the representation of the tradeoffs between sensitivity and specificity. The closer the graph follows the left hand border and then top border, the more accurate the test.

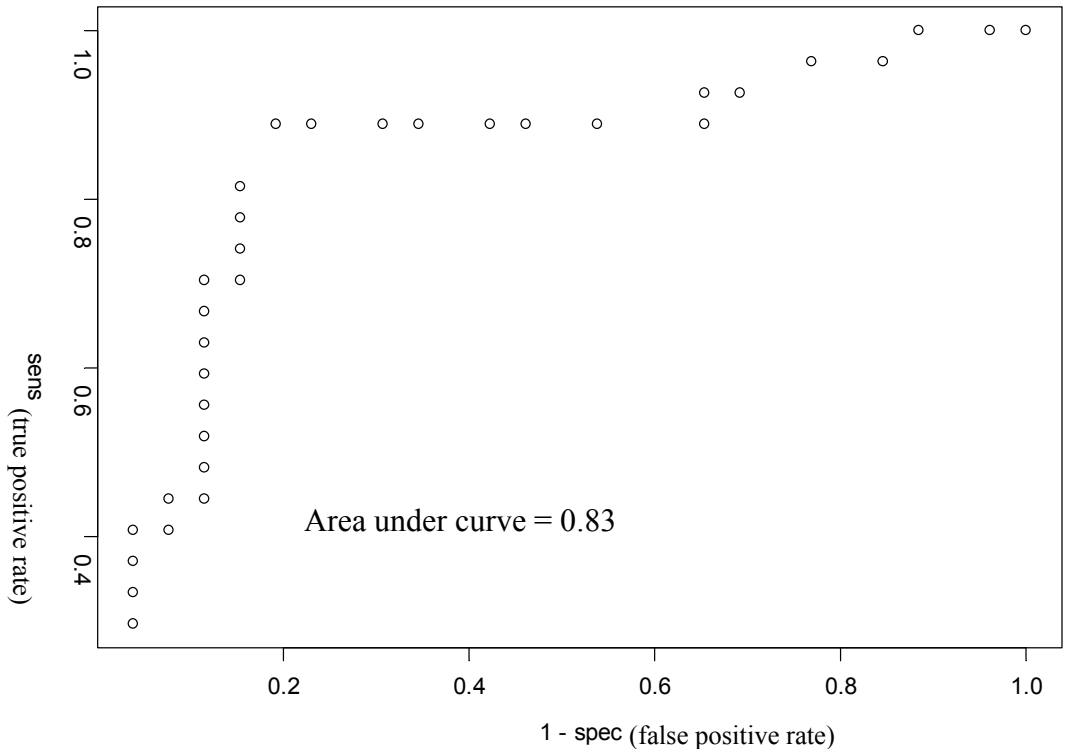

Figure 9: ROC curve

ROC curve plotting sensitivity by 1-specificity of the BLCA-1 urine-based immunoassay

The level of BLCA-1 is graphed according to tumor grade and there is not a significant correlation between tumor grade and BLCA-1 expression (Figure 10). BLCA-1 was detected in the urine from all three patients with low-grade disease. The mean absorbance for grade 1 tumors was 0.078, grade 2 was 0.041, and grade 3 was 0.084. More tumor samples, especially grade 1, must be collected to further delineate any correlation between urine BLCA-1 levels and tumor grade.

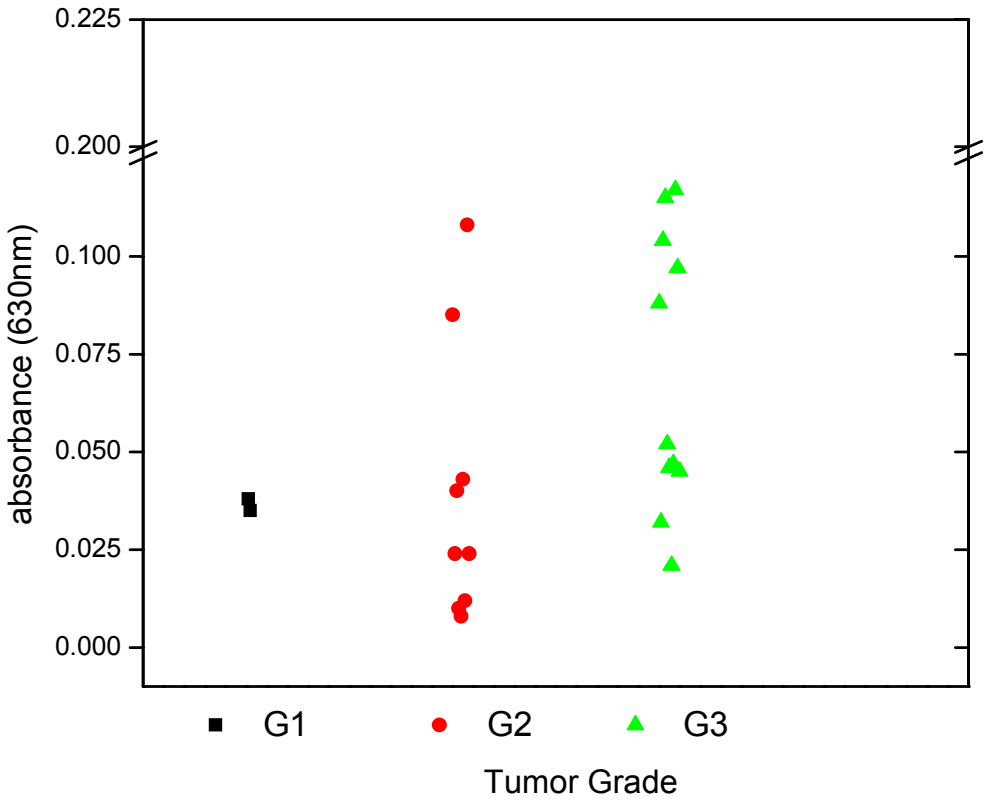

Figure 10: Urinary levels of BLCA-1 graphed according to tumor grade

Levels of BLCA-1 expression do not appear to correlate with tumor grade.

An ELISA was developed that is able to detect BLCA-1 in the serum of bladder cancer patients. After examining the data, there does not appear to be a correlation between metastatic disease and levels of BLCA-1. Because of difficulty obtaining metastatic serum samples, the sample number is very low. Using additional serum samples from individuals with bladder cancer we were able to identify a preliminary association between tumor stage and levels of BLCA-1 in patient serum (Figure 11).

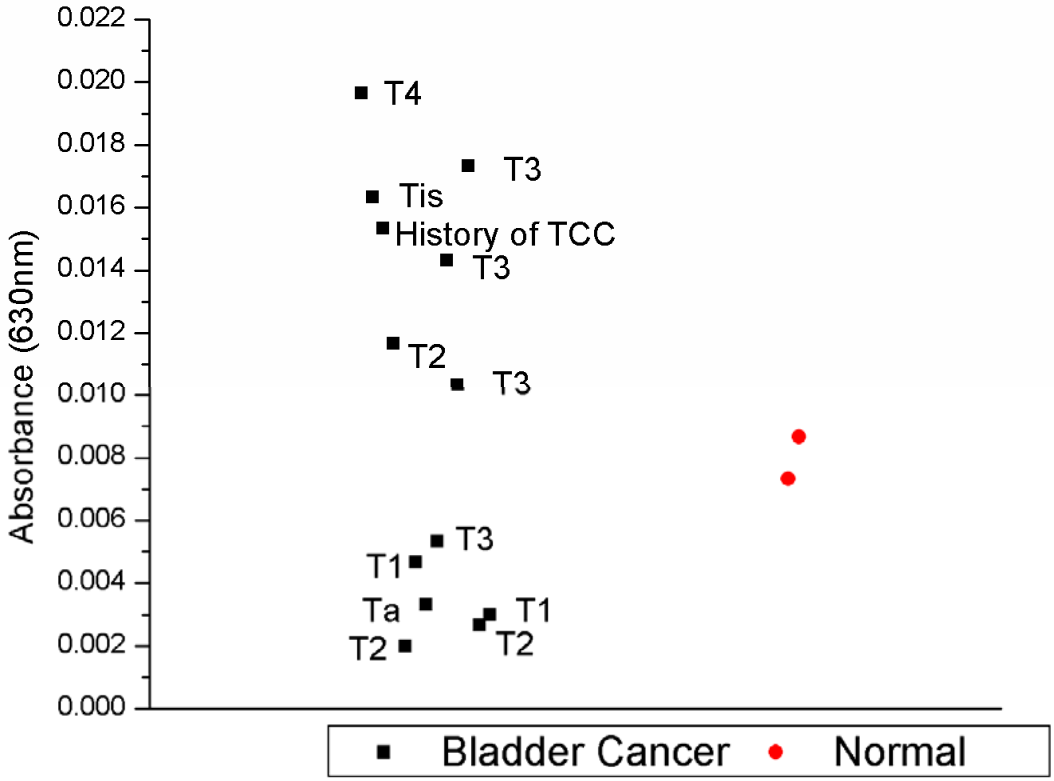

Figure 11: BLCA-1 serum based ELISA

A serum based ELISA assay can detect BLCA-1 in the serum of patients with high stage tumors.

6. Preliminary BLCA-1 Cloning Data

6.1. Introduction

BLCA-1 is a 72 kD nuclear matrix protein that was originally identified from bladder tumor tissue. We have been successful in isolating this protein and obtaining small peptide sequences. As discussed in chapter 4, based on peptide homology to known protein sequences, this protein has some potentially interesting motifs, although no functional information is currently available. We have also produced anti-BLCA-1 antibodies, which can detect BLCA-1 in bladder tumor tissue, but we do not see expression of this protein in normal tissue. Furthermore, we demonstrated that this protein is released into the urine and can be detected via immunoblots. We have also demonstrated our ability develop a urine-based immunoassay utilizing the bladder cancer specific nuclear matrix protein, BLCA-1.

In previous experiments we have displayed our ability to clone the cDNA encoding for the novel nuclear matrix protein, BLCA-4 (Van Le, et al. 2004). Utilizing degenerate primers from peptide sequences, a product was amplified, isolated, and cloned into a vector for sequencing. Analysis of the sequence data reveals that BLCA-4 shares a close homology to the ETS transcription factor family, as will be discussed in more detail in the following chapter (Van Le, et al. 2004). Further exploration of the functions of BLCA-4 is now possible.

The purpose of this study was to clone the cDNA that encodes for BLCA-1. Utilizing methods similar to that used to clone BLCA-4 we were able to successfully clone a portion of the cDNA that encodes for BLCA-1, although further confirmation is necessary.

6.2. Hypothesis

The gene encoding BLCA-1 reveals novel information in regards to the function of this protein in bladder cancer pathobiology

6.3. Results

Degenerate primers were produced to all of the peptide sequences obtained for BLCA-1. PCR was performed with various combinations of primers until a product was amplified. RNA was isolated from bladder cancer tissue or normal tissue and reverse transcribed to cDNA and used for PCR analysis. A band of approximately 500 bp was amplified utilizing bladder tissue cDNA and the following degenerate primer sequences (Figure 12):

Forward: 5' TACTTYAARCTYTACCTYGTYATRCANCTY

Reverse: 5' YCTYCTRCTYCTRAANCCYTCNAGNAGCCA

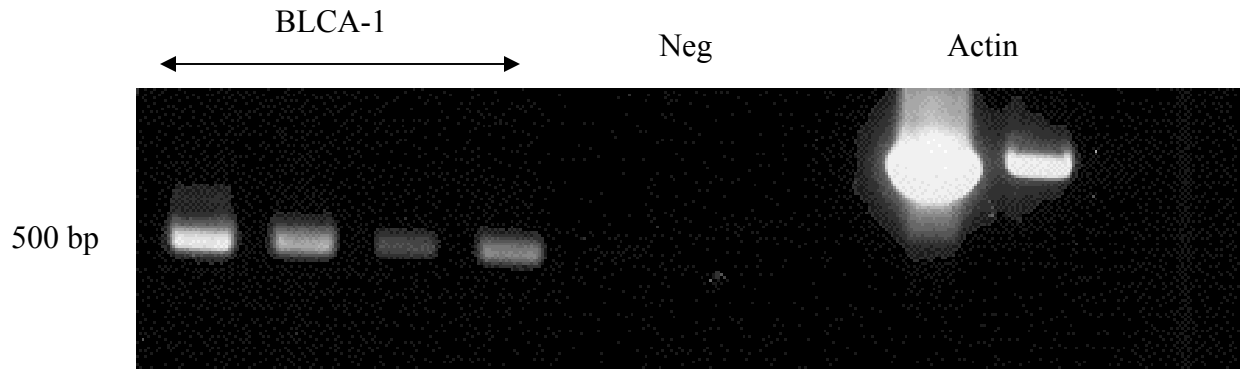

Figure 12: BLCA-1 PCR product

A product of approximately 500 bp was amplified using BLCA-1 degenerate primers.

DNA was isolated from the agarose gel and cloned into the pCR®4-TOPO® vector (Figure 13).

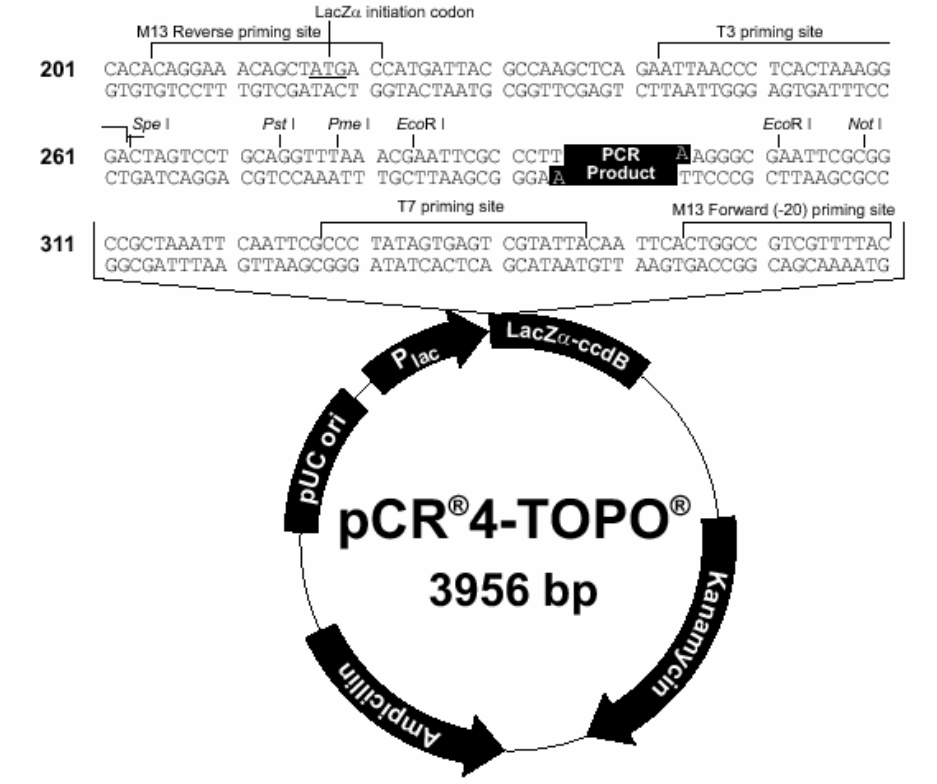

Figure 13: Map of pCR ®4-TOPO®

The map shows the features of pCR®4-TOPO® and the sequence surrounding the TOPO® Cloning site.

The vector containing the insert was cloned into chemically competent *E. coli* cells and plated. Transformed clones were selected based on resistance to ampicillin and color. White colonies indicate that the insert is present due to inactivation of the LacZ gene. Furthermore, The vector contains the lethal *E. coli* ccdB gene fused to the C-terminus of the LacZα fragment.

Ligation of a PCR product disrupts expression of the lacZαccdB gene fusion permitting growth of only positive recombinants upon transformation in TOP10 cells. DNA was isolated from cultures of cells containing the BLCA-1 insert. Verification of the BLCA-1 insert was completed by performing a restriction digest on the DNA. The EcoR1 enzyme was used to completely remove the insert from the vector (Figure 14).

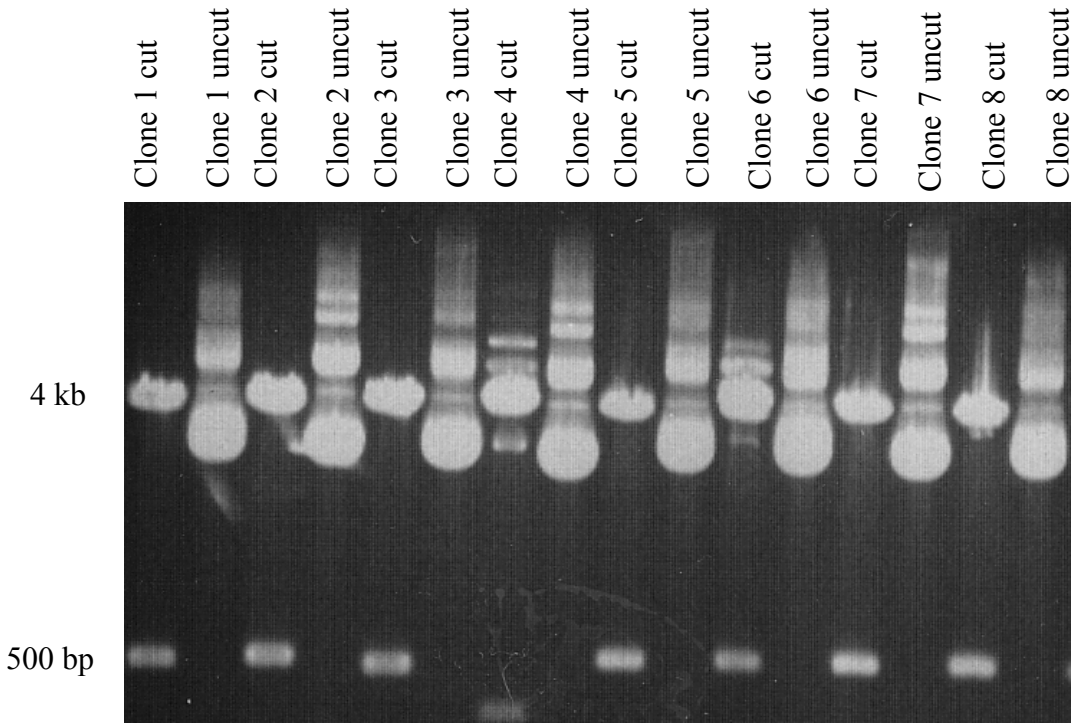

Figure 14: Restriction digest

A restriction digest was completed with EcoR1 to verify the presence of the BLCA-1 insert (500bp). Clones 1, 3, and 7 were used for sequencing.

DNA was isolated from the colonies verified to contain the BLCA-1 insert and sent for sequencing. Sequencing described a 516 bp fragment of DNA (figure 15). After analysis of the amino acid sequence we were able to identify a peptide sequence, LDQSITL, with close homology (5/7 amino acids) to one of the BLCA-1 peptide sequences, LDQDIDL (figure 15).

BLCA-1 cDNA sequence

```
ccccttccttctgcttctgaatccttcgagaagccaccaattaagaaagcgttcaagctcaaca
cccactacctaaaaaatcccaaacatataactgaactcctcacacccaattggaccaatctatc
accctatagaaaaactaatgttagtataagtaacatgaaaacattctcctccgcataagcctgt
gtcagattaaaacactgaactgacaattaacagcccaatatctacaatcaaccaacaagtcatt
attaccctcactgtcaacccaacacaggcatgctcataaggaaaggttaaaaaaagtaaaagga
actcggcaaatcttaccccgcctgtttaccaaaaacatcacctctagcatcaccagtattagag
gcaccgcctgcccagtgacacatgtttaacggccgcggtaccctaaccgtgcaaaggtagcata
atcacttgttccttaattagggacctgtatgaatggctcctagaaggtttcagaagagaaggaa
gggc
```

Translated amino acid sequence

```
PSFCF*ILREATN*ESVQAQHPLPKKSQTYN*TPHTQLDQSITL*KN*C*YK*HENILLRISLC
QIKTLN*QLTAQYLQSTNKSLLPSLSTQHRHAHKERLKKVKGTRQILPRLFTKNITSSITSIRG
TACPVTHV*RPRYPNRAKVA*SLVP*LGTCMNGS*KVSEEKEG
```

Figure 15: BLCA-1 gene and amino acid sequences

A 516 bp segment of BLCA-1 cDNA sequence was received and translated into an amino acid sequence.

The sequence obtained for BLCA-1 was compared with available databases and it appears to share a close homology (98%) to part of a novel metastasis associated gene, named TI-227H. The entire TI-227H gene is 1580 bp long and our sequence matches to 449 bp of the gene. Homology between the two sequences is shown in figure 16.

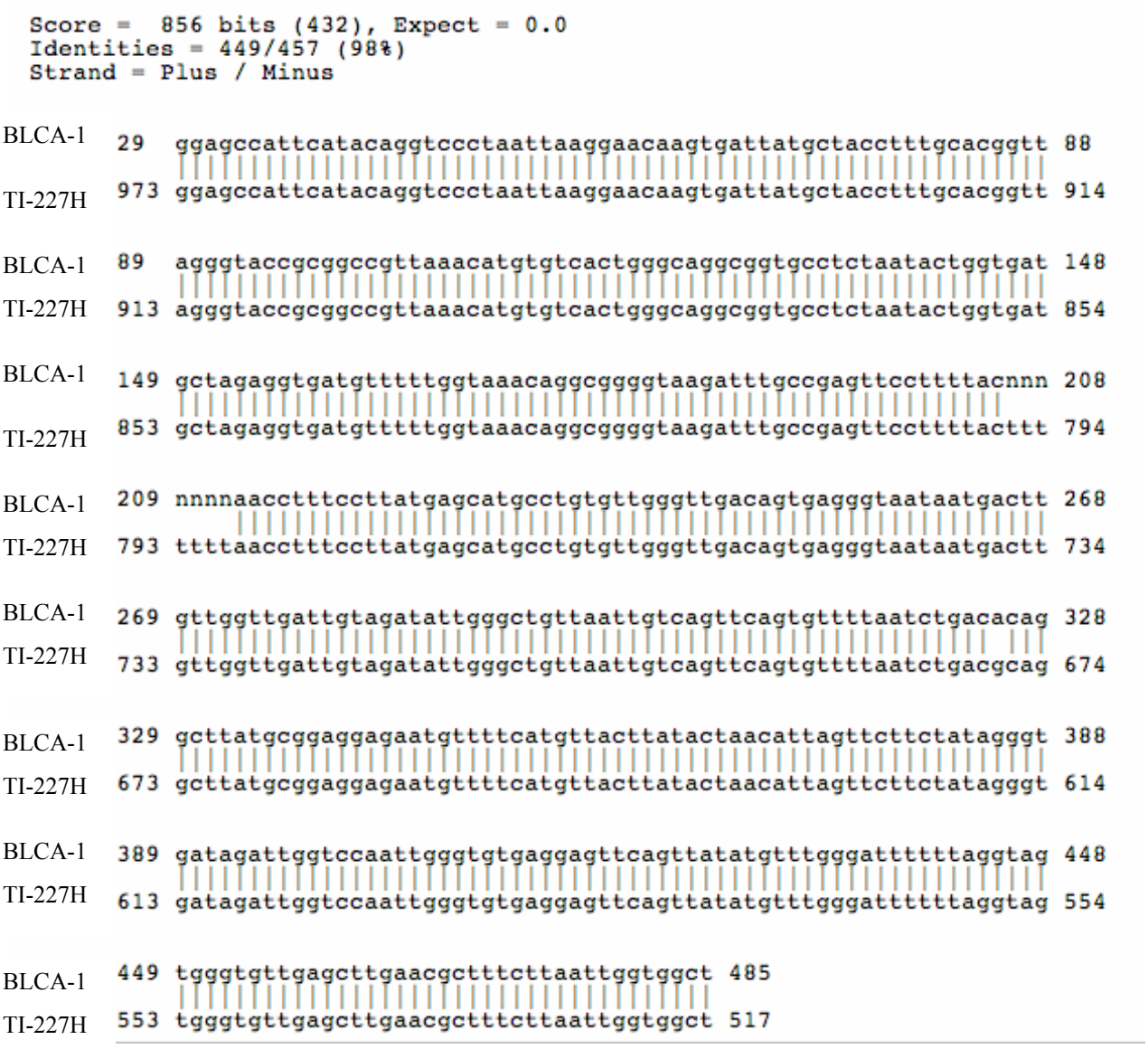

Figure 16: BLCA-1 BLAST results

The BLCA-1 sequence has a close homology to the novel metastasis gene, TI-227H.

7. Potential Roles of the Novel Nuclear Marker, BLCA-4, in Bladder Cancer Pathobiology

7.1. Introduction

Sequence data has been obtained from two of the more abundant bladder cancer specific nuclear matrix proteins, BLCA-1 and BLCA-4, which were identified by our lab. Antibodies to both of these proteins have been developed and it has been established that these proteins are detectable in both the tissue and urine of individuals with bladder cancer. Urine immunoassays have successfully been developed to both proteins. A sandwich immunoassay using BLCA-4 and a study of a large cohort of patients with bladder cancer, other benign urologic conditions, other cancer types, and normal controls, demonstrated a specificity of 95% and a sensitivity of 89% (Van Le, et al. 2004). A second immunoassay has been developed using an anti-BLCA-1 antibody and can detect bladder cancer from the urine of patients with a sensitivity of 80% and a specificity of 87% (Myers-Irvin 2005).

As mentioned in the previous chapter, the gene that encodes BLCA-4 has been identified and sequenced and has homology with the ELK-3 gene, a member of the ETS transcription factor family. ETS proteins have an ets domain that binds to DNA, specifically interacting with sequences that contain the sequence C/A GGA A/T. The ets domain is also involved in protein-protein interactions with co-factors that help determine its biological activity (Wasylyk, et. al 1993). The gene products of this family are transcription factors that control various cellular functions in cooperation with other transcription factors. The target genes for ETS transcription

factors include oncogenes, tumor suppressor genes, apoptosis-related genes, differentiation-related genes, angiogenesis-related genes, and invasion and metastasis-related genes (Oikawa 2004). Because these transcription factors are involved with so many biological functions, aberrant expression of these genes can contribute to malignant transformation and tumor progression. Specifically, ELK3 has been implicated in the promotion of angiogenesis and stimulation of VEGF expression (Zheng, et. al 2003). Preliminary work has been published demonstrating that BLCA-4 confers a growth advantage in T24 cells transfected with the gene encoding BLCA-4 when compared to vector only controls. In addition, it has been shown that BLCA-4 can interact with the ETS binding sequence as well as several transcription factors including, AP-1, AP-2, NFATC, NF-E1, and NF-E2 (Van Le, et al. 2004).

The purpose of this study is to further analyze the functional aspects of the BLCA-4 gene and its potential role in bladder cancer pathobiology. In order to accomplish this, the gene encoding BLCA-4 was transfected into a cell line that does not endogenously express BLCA-4. These cells were used to study cell growth as well as examine gene expression of the transfected and untransfected cells via microarray. A few genes found to be upregulated in the transfected cells were selected to further confirm their expression via immunoblot analysis.

7.2. Hypothesis

The nuclear structural protein, BLCA-4, which is specifically associated with bladder cancer plays a role in bladder cancer pathobiology.

7.3. Results

To begin to understand the function of BLCA-4 in bladder tumorigenesis, the gene encoding this protein was stably transfected into a human uroepithelial cell line (HUC cells). Transfected cells were treated with zeocin 48 hours after transfection and clones were picked two weeks later. The expression of BLCA-4 in several clones was analyzed by PCR using BLCA-4 gene specific primers. Four BLCA-4 over-expressing clones and one vector only clone were selected for future studies based on their BLCA-4 expression (Figure 17).

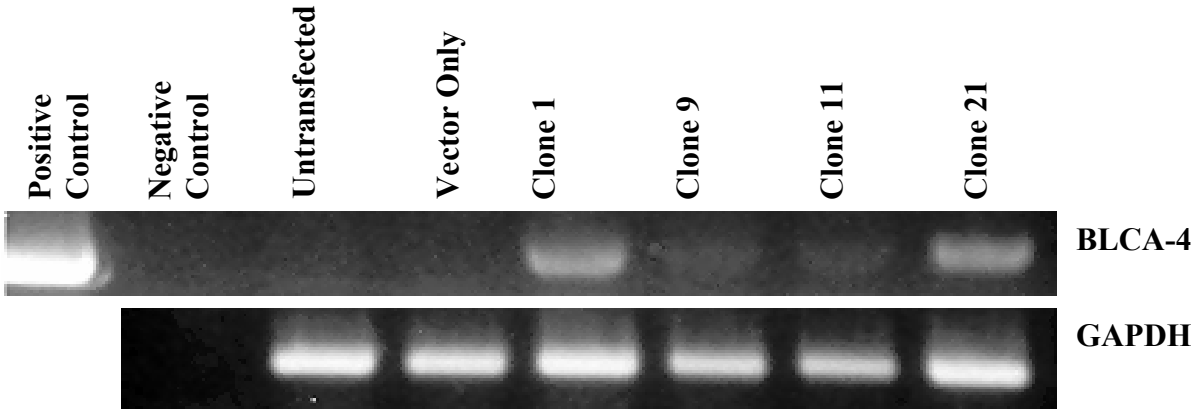

Figure 17: PCR analysis of BLCA-4 expression

BLCA-4 is expressed in the transfected clones 1, 9, 11, and 21, but is not expressed in the untransfected or vector only control cells.

Immunoblots were performed using whole cell lysates to examine BLCA-4 protein expression in the clones verified as expressing BLCA-4 at the message level. Clone 21 expresses the highest level of BLCA-4 protein, and lower quantities are produced by other clones (Figure 18). BLCA-4 expression was not examined in nuclear matrix preparations.

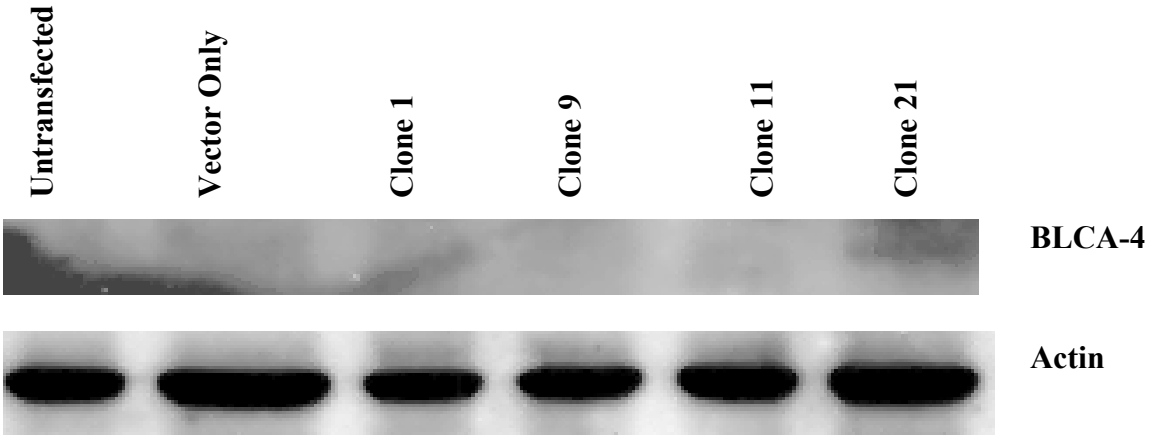

Figure 18: Immunoblot of BLCA-4 protein expression in transfected cells

BLCA-4 protein is most highly expressed in clone 21 and lower levels are detected in other clones, but BLCA-4 is not expressed in the untransfected cells or vector only controls.

In order to examine the role of BLCA-4 in cell proliferation, an MTT assay was performed on the transfected and control cells. Each timepoint for this assay is an average absorbance from eight wells, and this assay is representative of multiple experiments. Due to variations in absorbance readings, the values between experiments could not be combined, but the growth patterns were similar between experiments. The clones transfected with the gene encoding for BLCA-4 had a 4.3 fold greater proliferation rate than vector only controls or untransfected HUC cells (Figure 19).

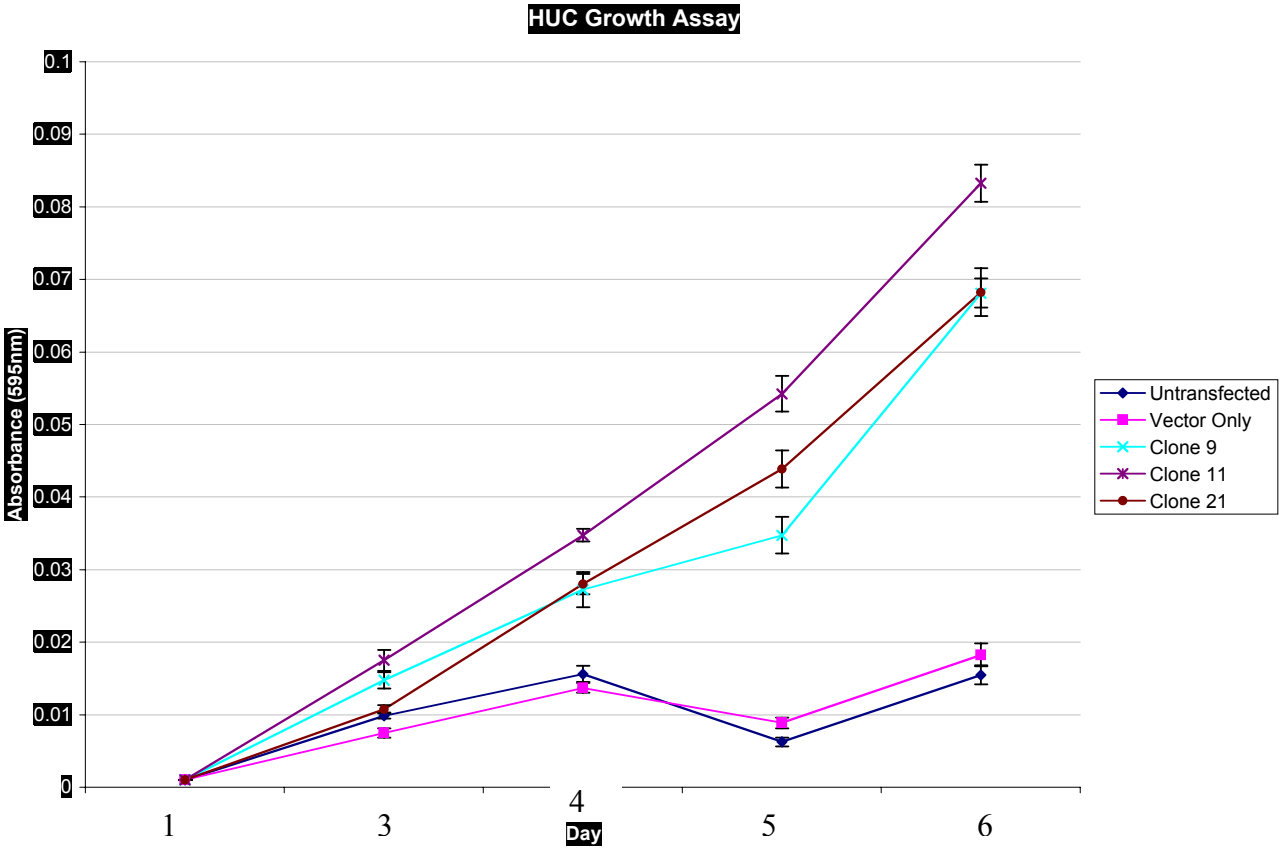

Figure 19: HUC cell proliferation assay

Cells transfected with the gene encoding BLCA-4 have a 4X higher growth rate than untransfected cells or vector only controls.

Cell counting assays were also performed to confirm the growth advantage in the transfected cells, and comparable growth patterns were observed (Figure 20).

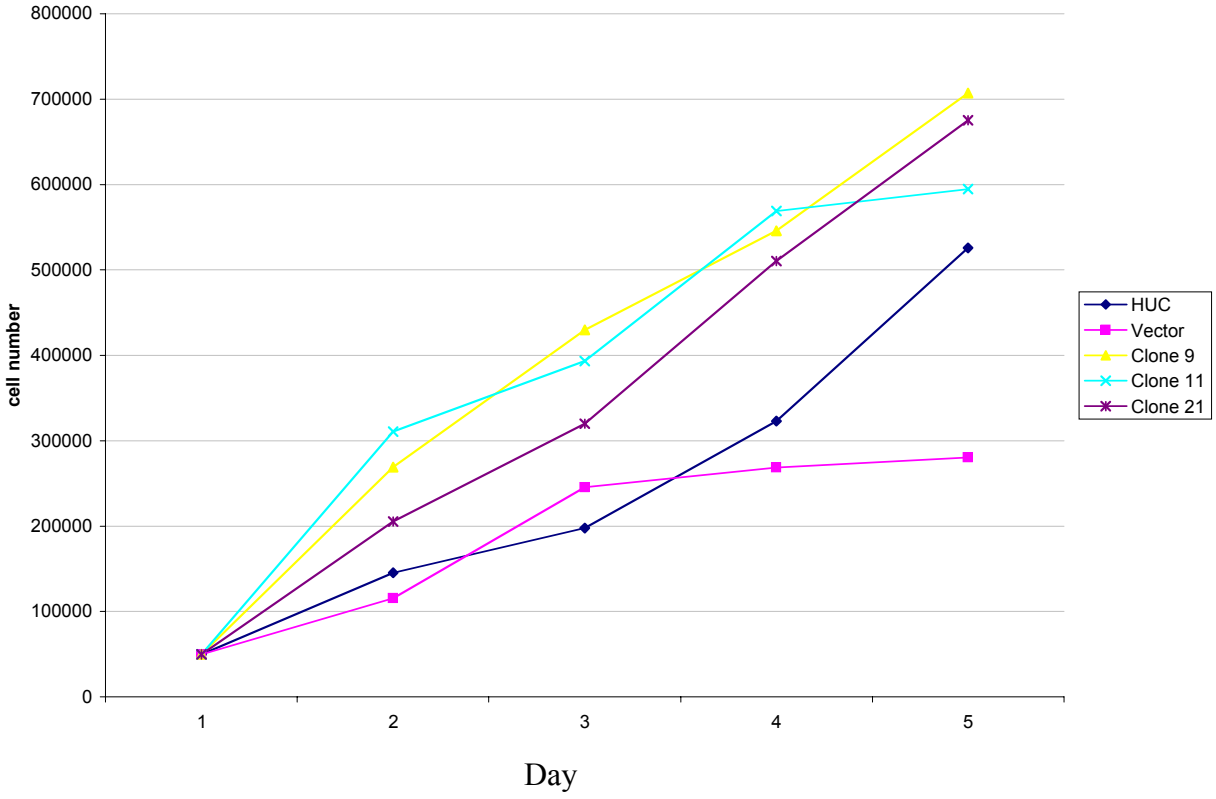

Figure 20: Cell counting assay

A cell counting assay was performed to confirm the increase growth rate in the transfected cells.

In order to examine alterations in gene expression that may be occurring as a result of BLCA-4 expression, gene expression patterns were compared between over-expressing and control lines. The BLCA-4 expressing clone, 21, was chosen for analysis due to its BLCA-4 expression as seen by PCR, western blot, and its increased proliferation rate. Approximately 38,500 genes were screened using Affymetrix U133 Plus 2.0 human genome chips. In order to choose genes of interest for further analysis, a cutoff of five-fold increase or decrease was chosen. It is acknowledged that this artificially defined cutoff will miss important changes of lower magnitude but this point was chosen to examine gross changes in expression (Tables 10 & 11).

Table 10: Genes that are downregulated in cells expressing BLCA-4 compared to vector only controls

	Vector Only Signal	Clone 21 Only Signal	Fold Change	Description
201009_s_at	641.3	53.5	12.5	upregulated by 1,25-dihydroxyvitamin D-3
206662_at	197.5	16.9	11.8	glutaredoxin (thioltransferase)
201010_s_at	757.9	71.3	10.5	upregulated by 1,25-dihydroxyvitamin D-3
214599_at	311.7	29.6	10.5	involucrin
202363_at	1269.3	131.4	9.7	testican-1 mRNA
203440_at	369.1	46.8	7.9	cadherin 2, type 1, N-cadherin (neuronal)
201008_s_at	774.7	107	7.2	upregulated by 1,25-dihydroxyvitamin D-3
207826_s_at	948.1	146.3	6.5	inhibitor of DNA binding 3, dominant negative helix-loop-helix
219274_at	132.8	22.2	6	transmembrane 4 superfamily member (tetraspan NET-2)
201426_s_at	2733	504.3	5.4	vimentin
205822_s_at	200.3	39.6	5.1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
209122_at	1826.5	368.8	5	adipose differentiation-related protein
207935_s_at	142.4	29.4	4.9	keratin 13

Table 11: Genes that are upregulated in cells expressing BLCA-4 compared to vector only controls

	Vector Only Signal	Clone 21 Signal	Fold Change	Description
210118_s_at	29	2066.3	71.3	interleukin 1-alpha
204614_at	23.3	1415.3	60.7	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2
204351_at	43.4	1409.9	32.5	S100 calcium-binding protein P
210544_s_at	47.8	1216.6	25.5	aldehyde dehydrogenase 10 (fatty aldehyde dehydrogenase)
208607_s_at	16.8	389.5	23.2	serum amyloid A2
203887_s_at	22.7	464.9	20.5	thrombomodulin
204470_at	49	876.3	17.9	GRO1 oncogene (melanoma growth stimulating activity, alpha)
203888_at	13.9	180.2	13.0	thrombomodulin
202859_x_at	271.4	3361.7	12.4	interleukin 8
211506_s_at	175.6	2010	11.4	interleukin 8 C-terminal variant
201110_s_at	64.4	632.5	9.8	thrombospondin 1
217999_s_at	18.9	185	9.8	pleckstrin homology-like domain, family A, member 1
210512_s_at	90.1	728.4	8.1	vascular endothelial growth factor
206864_s_at	9.1	72.9	8.0	harakiri, BCL2-interacting protein (contains only BH3 domain)
202644_s_at	154.1	1223.1	7.9	tumor necrosis factor, alpha-induced protein 3
201109_s_at	118.6	909.1	7.7	thrombospondin 1
205014_at	32.9	231.9	7.0	heparin-binding growth factor binding protein
214300_s_at	14.5	96	6.6	topoisomerase (DNA) III alpha

Clustering analysis was performed and a heat map of the genes was generated (Figure 21).

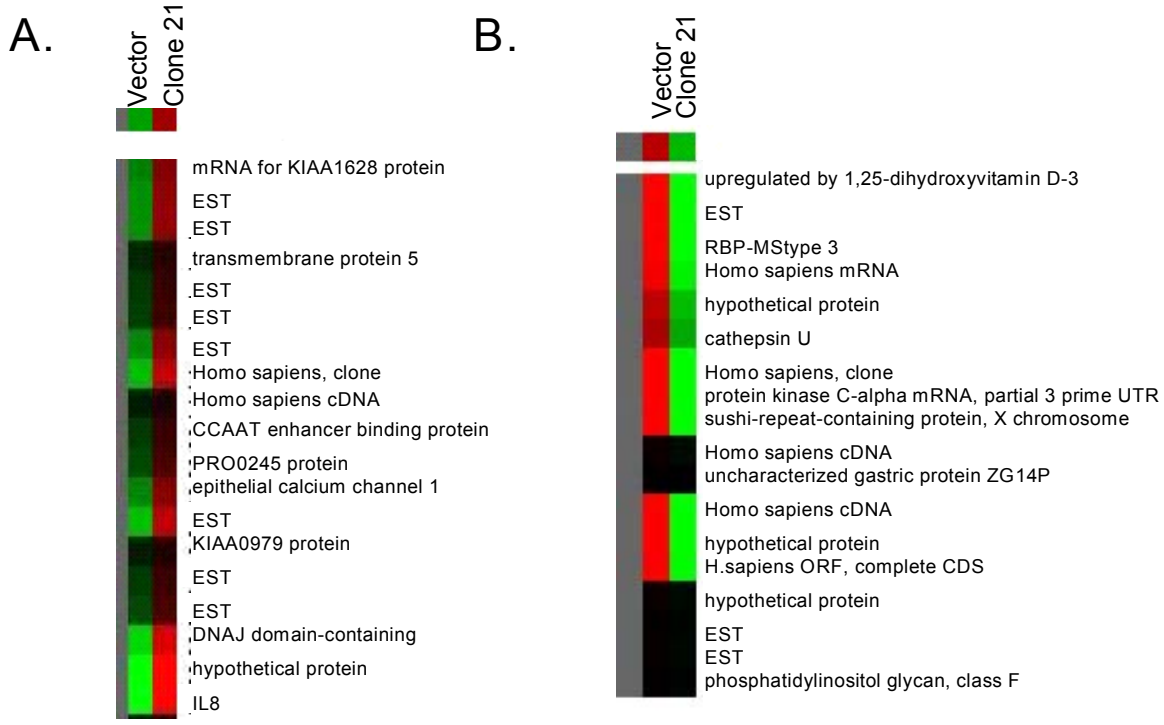

Figure 21: Examples of heat maps generated following clustering of genes.

A. Genes upregulated in clone 21compared to vector only clone. B. Genes downregulated in clone 21 compared to vector only clone

Western blot analyses of selected proteins were performed on whole cell lysates for further confirmation of the microarray data. The expression of interleukin 8, thrombomodulin, and interleukin1-α in whole cell lysates was examined by immunoblots. Expression of all three of these proteins correlated with the microarray data, as they were all over-expressed in the transfected clones compared to vector only controls (Figure 22). While the increase in IL8 may not be visibly obvious, densitometry indicates that the level of IL8 in clone 21 is increased over the vector only cells.

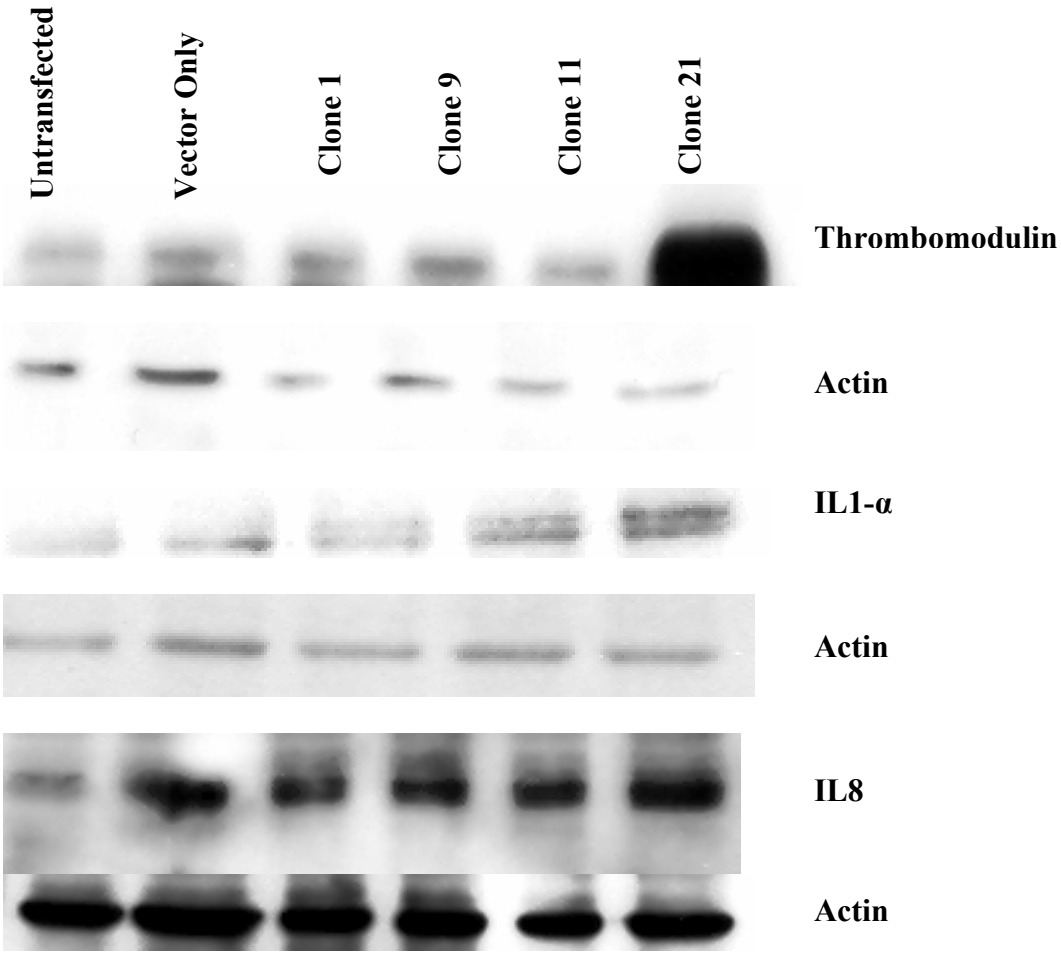

Figure 22: Western blot analysis of IL1-α, IL-8, and thrombomodulin

Cells transfected with BLCA-4 show increased expression of IL1-α, IL-8, and thrombomodulin. Clone 21 expresses the highest level of BLCA-4 and also expresses the highest amounts of IL1-α, IL-8, and thrombomodulin.

8. Discussion

Bladder cancer is a prominent disease affecting a large number of individuals each year. The current methods applied in the detection of bladder cancer lack sensitivity in the detection of low-grade tumors. It is very important to detect bladder cancer in the early stages, as the 5-year survival rate is 94% when this cancer is detected early at a localized stage (Society 2005). Cytology is commonly used to aid in bladder cancer diagnosis but lacks sensitivity. Cytology has a reported specificity of 94% but the sensitivity is only ~55% (Glas, et. al 2003). Low sensitivity is a significant shortcoming of this test as up to 45% of patients present with low grade tumors (Getzenberg, et al. 1996). The results of this test are also dependent on a pathologist's review and are not available immediately. The current "gold standard" to detect bladder cancer is cystoscopy combined with bladder biopsy, which is an invasive test that involves inserting a scope into the urethra. Because individuals diagnosed with bladder cancer have a high chance of recurrence (50-70%), they must be monitored frequently. After treatment, individuals must undergo a cystoscopic examination every three months for the first year, every six months for the second year, and then once a year annually after that (Sengupta, et al. 2004). The procedure is also expensive and can be questionable. Cystoscopy is difficult to perform on individuals with indwelling catheters and patients with bladder inflammation. This makes bladder cancer detection especially difficult in a very high risk population, people with spinal cord injuries. The lack of sensitivity of cytology and the invasiveness of cystoscopy render

detection of bladder cancer sub optimal. Therefore there is a great need for a test that is both sensitive and specific, noninvasive, inexpensive, and can easily be sampled over time.

Two urine-based tests are currently approved by the FDA for the detection of bladder cancer (Kantor, et al. 1984). As discussed earlier, these tests have their limitations as well. The reported sensitivity and specificity of these tests vary widely (Dey 2004). NMP22 detects a normal cellular protein that is over-expressed in individuals with bladder cancer. False-positive diagnoses may occur due to an increase in this marker in patients with inflammation, catheterization, urinary tract infections, and other urological diseases (Saad, et al. 2002). The BTA stat test can be performed quickly an easily, however the true sensitivity and specificity are again questionable. When tested on patients that have benign genitourinary conditions, the BTA stat test only has a specificity of 46%. Complement factor H is found in blood, so any individual presenting with hematuria may be diagnosed with a false-positive test (Simon, et al. 2003). These tests show more promise for the diagnosis of recurrent bladder cancer than detection of new disease.

The lack of an optimal diagnosis tool for bladder cancer has led our lab to explore new diagnostic tools. We have identified six nuclear matrix proteins that are specifically expressed in patients that have bladder cancer. We have previously demonstrated that BLCA-4 is expressed in urine of these individuals and can be detected using an indirect ELISA with a sensitivity of 96% and specificity of 100%, while the sandwich ELISA results in a sensitivity of 89% and a specificity of 95%. Furthermore, the expression of BLCA-4 is not affected by benign urinary conditions (Van Le, et al. 2004) and a large scale trial is now ongoing to confirm these studies. The levels of BLCA-4 are not associated with stage or grade of disease, and this protein can be

detected in individuals with low grade bladder cancer (Konety, et al. 2000). This nuclear matrix protein appears to be a promising biomarker for the diagnosis of bladder cancer.

Because of our success in creating an immunoassay using BLCA-4 to detect bladder cancer, we have decided to examine the potential of other bladder cancer specific nuclear matrix proteins to be used in a similar fashion. Nuclear matrix proteins were separated by 2D electrophoresis and spots corresponding to BLCA-1, 2, and 6 were isolated and sent for sequencing. Selected peptide sequences were conjugated to carrier proteins and sent for antibody production. Nuclear matrix proteins extracted from cancerous or normal bladder tissue were used to screen the BLCA-1, BLCA-2, and BLCA-6 antibodies for their ability to differentiate between disease states. Antibodies to all three proteins show initial promise in differentiating between cancerous and normal bladder tissue. The BLCA-1 antibody produced to the peptide WLLEGFRSRR demonstrated the best specificity and was used in assay development. While the antibodies to BLCA-2 and BLCA-6 have somewhat high backgrounds, they have shown promise in differentiating between donor bladder and cancerous bladder tissue. A large number of bleeds for both BLCA-2 and BLCA-6 have been collected but not screened. Based on initial testing, BLCA-2 and BLCA-6 may have the potential to be developed into specific markers of bladder cancer. Additionally, the cDNA for BLCA-2 and BLCA-6 will be cloned, which will allow us to examine the functional aspects of these proteins.

We have demonstrated that the BLCA-1 antibody can be used in immunoblots to selectively detect BLCA-1 both in tissue and urine of bladder cancer patients, while the antibody does not detect the protein in tissue or urine samples collected from normal donors. We have been successful in developing an immunoassay that can differentiate between urine samples from normal individuals and those with bladder cancer. This assay appears to be specific to bladder

cancer as the protein is generally not detected in the urine of individuals with prostate or renal cell carcinoma. While our antibody did detect a band in the tissue of renal carcinoma patients, it appears that this protein is not released into the urine, as it is not detected via immunoassay. We hypothesize that BLCA-1 is detected in renal tissue but not detected in the urine of patients with kidney cancer because of the amount of time the urine is in contact with the diseased tissue. While urine is filtered through the kidney, it has much more exposure to bladder tissue while in storage in this organ. We have previously seen this differential expression between tissue and urine with other proteins. Our immunoassay has slightly lower specificity than cytology, the currently used detection method, but the sensitivity is higher at 80%. Therefore, this assay may be clinically useful to increase the sensitivity of bladder cancer detection and as a result lead to increased survival of bladder cancer patients. One limitation of the study was the small number of low grade tumors. More low grade tumor urine samples will be collected to further evaluate any correlation with pathological grade and clinical stage. Three of the bladder cancer patients have been diagnosed with CIS. Because CIS is an aggressive tumor, these samples have been included in the grade 3 group and BLCA-1 was detected in all three.

Our lab has previously determined that BLCA-4 can also be selectively detected in tissue and urine from individuals with bladder cancer. While BLCA-1 is only detected in tumor tissue and not the normal adjacent or donor tissue, BLCA-4 is detected in both the tumor and normal adjacent tissue, suggesting this protein could be involved in a field effect for bladder cancer. Earlier research on BLCA-4 has also demonstrated that the expression of this protein does not vary between bladder cancer stages or grades. This study also suggests that there is not a statistically significant correlation between the level of BLCA-1 expression and the tumor grade. We do not have the information on the tumor stages for analysis at this point. Samples with

known staging information must be collected to examine an association between BLCA-1 levels and tumor stages. Future studies will be performed to examine the specificity and sensitivity of the BLCA-1 and BLCA-4 assays combined, as well as comparing the BLCA-1 assay to the NMP22 and BTA assays.

Metastasis of bladder cancer can occur in approximately 20% of patients with high-grade superficial disease. Invasion of tumor cells from one area to another requires the stimulation of new vascularization, proteolysis, increased cellular motility, proliferation, and escape from immune surveillance (Campbell, et al. 2002). This metastatic process requires tumor cells to progress through various stages, which may include alteration of gene expression or the products they encode for. Metastatic disease is difficult to identify and often times is not detected until visible symptoms are present. Therefore, metastatic markers would be useful so that aggressive treatments can be applied before tumors can spread. There has been research examining molecular markers that play a role in the progression of bladder cancer metastases. A few markers such as collagen IV, laminins, and E-cadherin have shown some potential as markers of metastasis (Gontero, et. al 2004). More research on these markers is needed before it can be determined if they can be used in daily clinical practice.

Our hypothesis is that BLCA-1 may be detectable in the serum of patients with bladder cancer and distinguish between those with localized and those with metastatic disease. If BLCA-1 is present in the serum, an assay may be developed that can distinguish between metastasizing and localized disease. More cells expressing BLCA-1 will exit from the bladder and enter into circulation, therefore allowing for detection of higher levels of this protein in the serum of individuals with metastatic disease. We were able to successfully develop an immunoassay, which detects BLCA-1 in the serum of bladder cancer patients. While the assay

was not successful in differentiating metastatic from localized disease, we did observe a preliminary association between serum levels of BLCA-1 and tumor stage. More serum samples must be collected before a definite association can be established. As mentioned in the introduction, tumor stage is a representation of the spread of the cancer. Higher stage tumors indicate that the cancer has spread out of the urothelium layer of the bladder into the muscle layer, fat layer, or beyond to distant sites. The bladder is a highly vascularized organ so one can see how cancer cells expressing BLCA-1 can enter circulation after breaking through the urothelium layer. BLCA-1 may still prove to be a marker of the aggressiveness of bladder cancer and therefore aid in treatment decisions for this disease.

As previously described, an antibody produced against the nuclear matrix protein BLCA-1 can selectively differentiate between both tissue and urine from individuals diagnosed with bladder cancer and normal donor patients as well as other control populations. This protein is a urine-based marker of bladder cancer that could be used in conjunction with the already developed BLCA-4 urine-based ELISA assay or alone to enhance sensitivity and specificity of the detection of this disease.

Despite much investigation, there remains little understanding of the basic biology of bladder cancer. After successfully developing immunoassays using the nuclear matrix proteins BLCA-1 and BLCA-4, we wanted to examine the mechanistic actions of these proteins in bladder cancer. While we have previously been successful in cloning the cDNA that encodes for BLCA-4, the cDNA sequence that encodes for BLCA-1 was unknown. By cloning the cDNA that encodes BLCA-1, we will be able to gather functional information regarding this gene by examining homology to known sequences as well as performing additional functional experiments that will be discussed in the future directions section of this thesis. In order to sequence the BLCA-1

cDNA, degenerate PCR primers were made based on the peptide sequences obtained. A ~500 bp product was amplified from bladder cancer tissue using a combination of two of the degenerate PCR primers. The DNA was isolated, cloned into a vector, and sent for sequencing. A 516 bp sequence was obtained for this fragment. This sequenced was compared to databases of known sequences, and results suggest that BLCA-1 may have a close homology to a novel metastasis gene, TI-227H.

Genes have been identified that are specifically associated with metastatic potential and behavior. Utilizing cell lines with differing metastatic potential, TI-227 was first identified in mouse melanoma cells. Human cDNA libraries were then used to isolate the human form of the gene. The Genbank/EMBL database was used to compare the gene sequence with known cDNA sequences, but no homologies were identified, indicating that TI-227H was apparantly a novel gene. Northern blot analysis was performed to identify TI-227H mRNA in normal tissue. Heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis tissue were screened, but TI-227H was not detected. TI-227H is found in mouse cell lines that metastasize preferentially to the lung (Ishiguro, et. al 2000). Upon gene sequence comparison, our BLCA-1 sequence has a close homology to a section of the TI-227H gene. Currently, no protein translation exists for the TI-227H gene. Based on the cDNA sequence it is estimated that the TI-227H protein would be 59 kD. We performed multiple frame shift translations of the TI-227H DNA and are able to identify one of our BLCA-1 peptides in one of the TI-227H amino acid translations. We also performed multiple frame translations of the BLCA-1 sequence and are able to identify the same peptide sequence as illustrated previously in figure 15. However, translation of the most obvious open reading frame in the TI-227H sequence with homology to our BLCA-1 data, does not reveal any peptide sequence matches. Our BLCA-1 sequences has a high degree of homology

with only part of the TI-227H gene. Our sequences matches with approximately one third of the sequence that encodes for TI-227H. Therefore, while we do not believe that BLCA-1 is the TI-227H gene, this preliminary data suggests that it may be related to a metastasis associated gene. Further confirmation of these results is necessary.

To determine the remainder of the specific sequence encoding BLCA-1, gene specific primers will be made to the BLCA-1 sequence that we obtained. These new primers will be used to screen for differential expression between normal and tumor bladder tissue. Furthermore, RACE (rapid amplification of cDNA ends) will be performed in order to extend the 3' and 5' ends of the sequence in order to obtain the entire cDNA sequence. Once the entire sequence is obtained it will again be compared to databases of known cDNA sequences to determine homologies. The BL CA-1 cDNA sequence will then be used to perform functional experiments to try to determine what role, if any, BLCA-1 has in the pathobiology of bladder cancer.

While we are still in the process of definitively cloning the cDNA that encodes for BLCA-1, we have successfully cloned the entire cDNA that encodes for BLCA-4. Utilizing this cDNA sequence, some of the functional aspects of BLCA-4 have begun to be elucidated. The cDNA was cloned and has been stably transfected into cell lines and it appears that BLCA-4 over-expression results in an increased growth rate in cells. Analysis of the gene sequence reveals that BLCA-4 shares a close homology with the transcription factor family of proteins. It specifically shares homology with the ELK-3 member of this family, and therefore may be a regulator of transcription. In addition, utilizing the TranSignal TF-TF Interaction Arrays I and II (Panomics, Redwood City, CA), it has been shown that BLCA-4 can interact with the ETS binding sequence as well as several transcription factors including, AP-1, AP-2, NFATC, NF-E1, and NF-E2 (Van Le, et al. 2004). Immunoblots of bladder tissue from mice treated with MNU to

induce bladder cancer demonstrate that BLCA-4 is expressed in the bladders of rats at weeks 8–34, as well as in the urine at weeks 2, 4, and 37 (the three time points tested) after MNU exposure. Results of the study indicate that the BLCA-4 antigen is absent in the normal rat bladder and urine but present in all of the bladders obtained from rats at each time point examined after MNU administration. The data indicate that BLCA-4 expression appears before the detection of grossly visible tumors in these animals. Therefore, BLCA-4 may be an early onset marker in the development of bladder cancer (Van Le, et al. 2004). It is unclear how early in the cancer process BLCA-1 is expressed. Animal studies, in which bladder cancer is induced, can be used to test how early this protein appears in bladder cancer progression.

It is recognized that the nuclear matrix plays a role in many functions that are implicated in cancer progression and it has been established that the nuclear matrix is altered in cancer cells. Therefore, changes in this structure may correspond to changes in nuclear shape, the hallmark of a cancer cell and with alterations of nuclear processes. The purpose of the studies performed here was to further understand the role of the novel nuclear matrix protein, BLCA-4, in bladder cancer by altering expression levels of this gene in a cell model and examining the effects of this over-expression. We have transfected the gene that encodes for BLCA-4 into a "normal" immortalized non-tumorigenic cell line that does not express BLCA-4. We used the HUC, or human uroepithelial cell line for these experiments. Stable transfectants were chosen and analysis demonstrated over-expression of BLCA-4 at the message and protein levels. Based on these analyses, clones were selected to test for cell proliferation and the transfected clones had a significantly higher growth rate. Introduction of BLCA-4 therefore appears to confer a growth advantage for cells. While clone 21 expressed the highest level BLCA-4 protein, it had the same growth rate as clones 9 and 11, which expressed lower levels of BLCA-4. It is possible that any

expression of BLCA-4 protein is enough increase the growth rate in cells. In the future, it would be interesting to examine if there is a threshold level of BLCA-4 which must be reached in order to confer a growth advantage in cells.

An *in vivo* tumoirgenicity assay was performed utilizing the transfected HUC cells. The right hind legs of nude mice were injected with 250,000 cells that over-express BLCA-4 (clone 21), while the left hind legs were injected with 250,000 vector only control cells. The experiment was carried out for six months, but not tumor growth was observed with either cell line. We believe that our cell load was too low, and in the future we plan on increasing the number of cells that are injected. Additionally, in the future we can use Matrigel in order to aid in cell establishment in the mice.

Analysis of microarray data was performed to further explore some of the genetic changes that may be occurring as a result of BLCA-4 expression. Microarray analysis was completed comparing cells over-expressing the gene encoding BLCA-4 to cells transfected with vector alone. Our results show a large number of genes that are either upregulated or downregulated in the cells expressing BLCA-4 in comparison to the vector only controls. Because of the considerable number of genes that were altered in the BLCA-4 expressing clones, a cutoff of 5-fold increase or decrease was used to filter out genes for further analysis. Many genes that are altered as a result of BLCA-4 expression have implications in cancer progression or as markers of disease. Three genes that are upregulated in the BLCA-4 expressing clones, interleukin-1α, interleukin-8, and thrombomodulin, were chosen for further analysis based on their level of expression and availability of commercial antibodies for use in the confirmation studies.

IL-I is a major cytokine in the generation of an inflammatory response. There are two isoforms of this protein, IL-1α and IL-1β, although both forms seem to have very similar biological functions. Some effects of IL-1 in tumors have been reported. It appears that this protein can have both pro- and anti-tumoral effects (Seddighzadeh, et. al 2001). IL-1 can have growth inhibitory effects on tumor cell lines, has exhibited anti-angiogenic properties, and can also upregulate host defenses and function as an immunostimulatory agent. However, it has also been reported that in other cases IL-1 may advance metastasis by promoting tumor cell-endothelial cell adhesion and can also increase expression of matrix degrading enzymes such as uPA and stomelysins (Seddighzadeh, et al. 2001). Our microarray study demonstrated a marked increase in IL-1α expression in cells transfected with the BLCA-4 gene. This increased expression was also verified at the protein level by immunoblots. There is not much literature available at this time exploring the expression of IL-1α in bladder cancer. However, the increase in IL-1α expression may be increasing the tumorigenic potential of the cells expressing BLCA-4.

Interleukin-8, first identified as a leukocyte chemoattractant, is known to induce angiogenesis and is expressed by various tumors, including transitional cell carcinomas of the bladder. Expression of IL-8 also correlates with the metastatic potential of tumors (Inoue, et. al 2000). It has been discovered that levels of IL-8 are elevated in the urine from individuals with transitional cell carcinoma of the bladder (Sheryka, et. al 2003). Additionally, human anti-interleukin 8 antibodies have demonstrated the ability to inhibit tumor growth *in vivo* (Mian, et. al 2003). Our microarray analysis demonstrated a 12-fold increase in the expression of IL-8 in the clone over-expressing BLCA-4, demonstrating the potential role of BLCA-4 in increasing tumorigenicity of bladder cancer.

An additional gene of interest that is upregulated in the transfected clone is thrombomodulin. Thrombomodulin is a transmembrane glycoprotein that is involved in regulating intravascular coagulation. The expression of thrombomodulin in bladder cancer has not been studied extensively. However, it has been shown that thrombomodulin can be a sensitive but nonspecific marker of urothelial carcinoma (Parker, et. al 2003). Plasma levels of thrombomodulin have been reported to increase with progression of malignant disease of various cancers (Iqbal 2000).

There are other interesting genes to note that are upregulated in the BLCA-4 expressing cells that were not further confirmed at the protein level by immunoblots and may have a potential impact on bladder cancer progression. Vascular endothelial growth factor is an angiogenic agent that has been reported to be upregulated in a multitude of tumors. Serum amyloid is a cytokine that is increased as a result of inflammation or injury and can also influence cell motility, adhesion, and proliferation and may be a potential serum marker of various diseases, including lung cancer (Khan, et. al 2004). Genes found to be downregulated in the BLCA-4 transfected cells were also analyzed. Vitamin D3 upregulated protein was downregulated in our comparison. It is found to be downregulated in some tumor types and has been suggested to have tumor suppressor activity (Han, et. al 2003). There are a great number of genes that are altered by BLCA-4 expression that can be further explored as components of other studies.

Some of the potential pathways through which BLCA-4 aids in the pathobiology of bladder cancer are outlined in Figure 23. BLCA-4 over-expression has been shown to upregulate IL1-α. IL1-α in turn can increase the expression of matrix degrading enzymes as well as promote tumor cell endothelial cell adhesion. The result of over-expression of IL1-α could ultimately lead to enhanced proliferation and invasion of bladder tumor cells. BLCA-4 over-expression also

results in increased expression of thrombomodulin, an anticoagulant. Tumor vasculature is leaky and can be prone to being plugged with blood clots, thus blocking blood flow to the tumor. Anticoagulants aid in maintaining the blood flow necessary for tumor cell survival. Additionally, over-expression of BLCA-4 leads to increased expression of IL8. Research indicates that IL8 increases angiogenesis, therefore providing necessary blood flow to tumors. While we noted that an increase in BLCA-4 expression leads to an increased growth rate in cells, the genes we choose for further analysis are related to tumor metastasis and progression. While BLCA-4 may not directly cause an increased expression of these genes, it may be a downstream effect of BLCA-4 over-expression. Microarray analysis indicates that there are genes related to cell growth, such as the cyclins, that are upregulated in the cells over-expressing BLCA-4 and can be further explored in the future. Analysis of the mRNA of IL-1α, IL-8, and thrombomodulin reveals that each gene has multiple ETS sites in their sequence. We have previously demonstrated that BLCA-4 has the ability to bind to ETS sites. Therefore, it is possible that BLCA-4 is binding directly to each gene to cause the increase in expression.

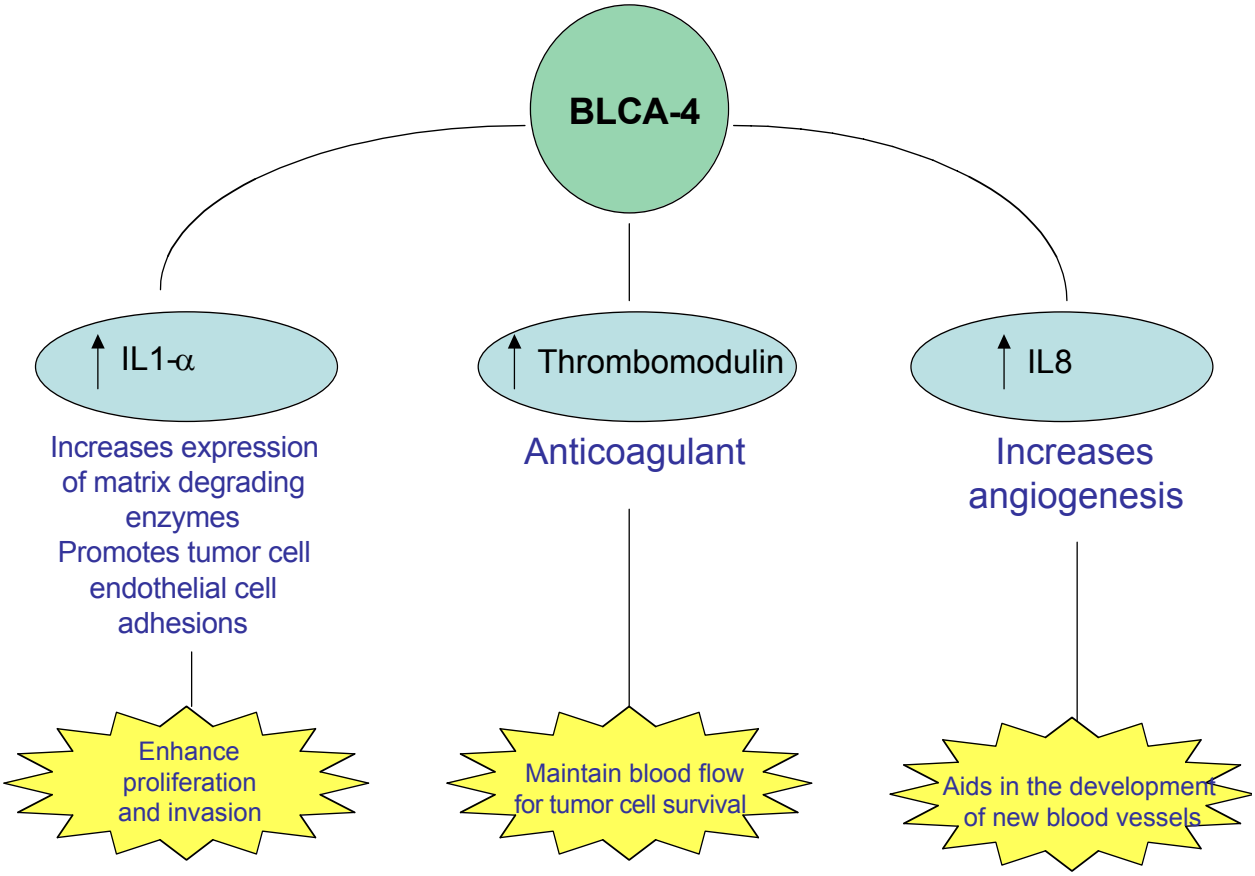

Figure 23: Model of potential BLCA-4 action

The studies outlined in here provide more insight into the nuclear matrix protein, BLCA-4, which has already shown great promise as a marker of bladder cancer. We have shown that over-expression of BLCA-4 can successfully be achieved by stable transfection into bladder cells and confers a growth advantage in this cell model. We have also begun to elucidate some of the possible roles that BLCA-4 may play in the pathobiology of bladder cancer by performing microarrays. As a result of this analysis, a number of genes that are involved in cancer progression have been found to be altered by BLCA-4, and may be targets for future research. It is interesting to note that clone 21 expresses the highest level of BLCA-4 at both the message and protein levels, and also shows the most noticeable increase in IL-1α, IL-8, and thrombomodulin expression. While we were able to detect the BLCA-4 protein in other clones, it was at a much lower level than clone 21. Furthermore, the protein level increase of IL-1α, IL-8, and thrombomodulin is not as apparent in these other clones. Therefore, we hypothesize that the level of BLCA-4 protein expression may be affecting the levels of the proteins we examined. It appears that expression of BLCA-4 not only increases the growth rate in cells, but also creates a more tumorigenic phenotype. Additional studies will be performed in animal models to examine the tumorigenic potential of BLCA-4.

9. Conclusion

Nuclear matrix proteins are the scaffold of the nucleus and have a variety of functions, many of which are implicated in tumorigenesis. A few of the functions of the nuclear matrix include DNA organization, stabilization, and orientation during replication, determination of nuclear morphology, organization of gene regulatory complexes, and synthesis of RNA (Konety, et al. 1999). The studies outlined above demonstrate that these proteins are able to be developed into markers of specific diseases. Furthermore, they have the ability to interact with transcription factors and increase growth rate in cells. Expression of these proteins can alter gene expression in a cell model. This therefore demonstrates not only their diagnostic potential, but their therapeutic targets and prospective roles in the pathobiology of disease.

10. Future Directions

10.1. Optimization of BLCA-1 urine-based assay

Preliminary data demonstrates that an antibody raised to BLCA-1 can differentiate between individuals with bladder cancer and those without, with a specificity of 87% and a sensitivity of 80%. A higher sensitivity and specificity will improve the clinical significance of this immunoassay. In order to optimize this assay both the primary and secondary antibody concentrations can be varied individually to find the optimal concentrations of each. Currently the antibodies are each incubated for 2 hours at room temperature, but the time of antibody incubation can be altered if necessary. Another step which can be optimized is the detection time. The plates can be read at various timepoints to determine the most favorable development time. We are also in the process of producing BLCA-1 monoclonal antibodies. These monoclonal antibodies may be useful to produce a sandwich based ELISA and potentially increase the sensitivity and specificity of the assay. While our data show promising utility of BLCA-1 as a urine-based marker of bladder cancer, large scale studies must be performed following optimization in order to validate the findings.

Our lab has been successful in developing two urine-based immunoassays for the detection of bladder cancer, using novel nuclear matrix proteins expressed in the urine. Large-scale clinical trials with the BLCA-4 immunoassay have produced an assay with 95% specificity and 89% sensitivity. A study we wish to perform in the future is to explore the use of the BLCA-4

and BLCA-1 immunoassay combined. A combination of markers will provide the best clinical utility.

10.2. Additional BLCA-1 Characterization

In the future, we would also like to do some additional characterization of BLCA-1. The information we currently have does not indicate at what point BLCA-1 appears during the development and progression of bladder cancer. In the future we would like to establish the timing of BLCA-1 expression. In order to accomplish this we can use an animal model of bladder cancer. It is difficult to use human subjects for this research because the current detection methods make bladder cancer difficult to detect early. By using the animal model we can examine BLCA-1 expression in both tissue and urine from initiation of bladder cancer to late stage bladder cancer. In order to do so, bladder cancer can be induced in rats by administering MNU (N-Nitroso-n-Methylurea) intravesically into the bladder. The rats would be sacrificed at various timepoints and BLCA-1 levels examined.

In addition to examining the timing of BLCA-1 expression, we could examine BLCA-1 levels following bladder cancer therapy. This will allow us to determine if BLCA-1 expression levels are altered by surgery or intravesicular therapy, and therefore establish its utility as a marker of recurrence as well as therapy efficacy. In order to do so, samples will be collected from individuals before undergoing a TURBT. Because standard follow-up requires frequent visits to the urologist, urine samples can be collected and examined for BLCA-1 expression. Similar research could involve examining BLCA-1 expression following tumor resection and intravesical therapy for bladder cancer. Urine samples can be collected before treatment, and then again after resection. Samples can be collected before each intravesical treatment and again at the end of therapy. These experiments would allow us to determine if BLCA-1 is able to

detect recurrent cancer. These are important questions to answer as bladder cancer has a high rate of recurrence and cytology is not an optimal assay and cystoscopy is expensive and invasive.

10.3. Functional Roles of BLCA-1

Our knowledge of the role of BLCA-1 is currently limited. In order to examine the role of BLCA-1 in the pathobiology of bladder cancer, we can perform experiments similar to the ones we performed with BLCA-4. The gene that encodes BLCA-1 will be transfected into cells in order to alter expression of BLCA-1. HUC cells could be used because initial studies show no expression of BLCA-1 in these cells. In addition, expression could be altered by using the siRNA method to reduce or eliminate BLCA-1 expression. Initial results indicate that T24 express endogenous levels of BLCA-1 and this cell line would therefore be used for the siRNA experiments. Following altering the expression of BLCA-1 experiments can be done to examine the effect on cell morphology and proliferation. Morphology would be examined by viewing the cells under a microscope. Cell proliferation could be examined by doing cell count experiments or using an MTT assay. Furthermore, we propose to examine the invasive potential of BLCA-1 by examining its growth through a solid gel of membrane proteins as well as examining the effect of BLCA-1 on clonogenecity.

Once BLCA-1 expression has been established in cells, we would propose to do *in vivo* experiments and look at the ability of the cell lines to induce tumors in nude mice. Cells can be injected subcutaneously on each leg, or alternatively, a Matrigel solution could be used. A more complex approach would involve orthotopoic injection of the cells into the bladder. These studies are important to examine the tumorigenicity of BLCA-1. These same experiments can be proposed to examine the tumorigenicity of BLCA-4, as this has not yet been examined.

Our studies indicate that BLCA-1 is a novel protein but may be related to a metastasis associated gene. In order to rule out that BLCA-1 is a post-translationally modified pre-existing protein and further confirm that BLCA-1 is indeed a novel protein, we could examine post-translational modifications. Cells can be grown in the presence of radiolabeled ATP and then the isolated NMPs run on a 2D gel. Likewise, NMPs can be dephosphorylated and then run on 2D gels. Changes in mobility of BLCA-1 will be noted if this protein is phosphorylated. Moreover, we can propose to examine glycosylation of the protein. If we determine that BLCA-1 is phosphorylated or glycosylated, it does not mean that BLCA-1 is not a novel protein. These experiments will allow us to differentiate between BLCA-1 being post-translationally modified and BLCA-1 being the result of a post-translationally modified pre-existing protein.

Antibodies can also be used to examine some of the functional aspects of BLCA-1. We have successfully generated antibodies to both of these proteins. These antibodies can be used to perform immunoprecipitations in order to examine protein interactions. We could obtain valuable information about the role of BLCA-1 by identifying proteins that immunoprecipitate with antibodies raised to BLCA-1.

We have previously demonstrated that BLCA-4 can bind to DNA and specifically interacts with transcription factors. We could therefore propose to examine DNA binding studies with BLCA-1. The nuclear matrix has been shown to bind to DNA in regions termed MARs. We could therefore use some of the most common MAR sequences to examine their interactions with BLCA-1 via electromobility shift assays. Some examples of the MAR sequences we will would examine are the MAR which had originally been termed SAR from the Drosophila histone gene repeat, the 5' MAR of the chicken lysozyme gene, and MAR sequences from the mouse immunoglobulin kappa light chain locus. The experiments we have completed using BLCA-1

and BLCA-4 have produced very interesting results and promises to further our understanding of bladder cancer pathobiology as well as aid in the diagnosis of the disease. However, as represented here, there is still much to learn about these novel nuclear matrix proteins.

10.4. Long Term Objectives

Our vision for the BLCA-1 and BLCA-4 immunoassays is a point of care test that is administered to individuals during their physical exams. This test could be especially useful to monitor high-risk individuals such as those that smoke or patients with spinal cord injuries. Ultimately, assays using both proteins would be desired, as multiple markers could potentially increase the sensitivity and specificity of diagnosis. Depending on results of further studies, these tests may also be adapted to monitor recurrence in individuals previously treated for bladder cancer. Our research has the potential to have a large impact on the field of bladder cancer diagnosis and our understanding of this disease.

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